A LABORATORY MANUAL

OF

SOIL BACTERIOLOGY

BY

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PREFACE

THE exercises described in this laboratory manual are arranged primarily for students of soil bacteriology, soil chemistry and physics, and plant pathology. As far as possible the experiments are planned to give quantitative results. It is assumed that the student has had previous training in general bacteriology and chemistry.

The section entitled "Formulæ and Methods" is intended to present in a convenient form some of the more important media and methods used in a study of soil bacteria. The chemical methods employed are, with few exceptions, those given in standard text-books. Much of the material was collected and arranged by Mr. C. Hoffmann.

In addition to the references given in the text, frequent use has been made of the various manuals in bacteriology.

Any suggestions in regard to improvement of the manual will be welcomed.

E. B. FRED.

MADISON, WISCONSIN,
October, 1916.

CONTENTS

SOIL BACTERIOLOGY

INTRODUCTION

APPARATUS FOR ONE STUDENT

THE following apparatus should be in each desk. Any omission must be reported to the instructor at once.

1	Bunsen burner and tubing	$ .40
2	Wire baskets	.50
1	Metal cup	.25
2	4-inch funnels	.24
1	Graduate cylinder (100 c.c.)	.35
2	Erlenmeyer flasks (1000 c.c.)	.80
4	Erlenmeyer flasks (500 c.c.)	1.00
2	Erlenmeyer flasks (150 c.c.)	.30
50	Test-tubes (small)	.75
5	Test-tubes (large)	.15
5	Petri dishes	1.00
20	Glass tumblers	1.00
10	Pipets (1 c.c.)	.80
2	Pipets (10 c.c.)	.30
1	Hanging-drop slide	.20
1	Thermometer	.75
1	Platinum needle	.35
50	Object slides (not returnable)	.50
50	Cover glasses (not returnable)	.25
1	Aluminum weighing dish	.20
6	Evaporating dishes	.60
1	Test plate	.20
1	Wash bottle	.10
	Filter-paper (8-inch)	.10
1	Forceps (steel)	.25
1	Spatula	.25
1	Trowel	.10
1	Mixing cloth	.10
1	Slide box	.10
1	Test-tube brush	.05
1	Towel	.05
1	Box of matches	.01
1	Box of labels	
		$12.00

LITERATURE

The following list includes some of the more important books and journals that treat of bacteriology:

A. *General Bacteriology:*

Benecke, W.....Bau und Leben der Bakterien, 1912.
Charpentier, P. G.....Les Microbes, 1909.
Frost and McCampbell....Text-Book of General Bacteriology, 1912.
Hiss and Zinsser.....Text-Book of Bacteriology, 1915.
Jordan, E. O.....General Bacteriology, 5th ed., 1916.
Kayser, E.....Microbiologie Agricole, 3d ed., 1914.
Kruse, W.....Allgemeine Mikrobiologie, 1910.
Meyer, A.....Die Zelle der Bakterien, 1912.

B. *Agricultural Bacteriology:*

Conn, H. W.....Agricultural Bacteriology, 2d ed., 1909.
Fuhrmann, F.....Vorlesungen über Technische Mykologie, 1913.
Kossowicz, A.....Einführung in die Agrikulturmykologie, Teil I, Bodenbakteriologie, 1912.
Lipman, J. G.....Bacteria in Relation to Country Life, 1911.
Löhnis, F.....Vorlesungen über landwirtschaftliche Bakteriologie, 1913.
Marshall, C. E.....Microbiology, 1911.
Percival, J.....Agricultural Bacteriology, 1910.
Russell and Hastings.....Agricultural Bacteriology, 1915.

C. *Reference Books in Agricultural Bacteriology:*

Duclaux, E.....Traité de Microbiologie, 1898–1901.
Lafar, F.....Handbuch der Technischen Mykologie, Bd. III, 1904–1906; Mykologie des Bodens, des Wassers und des Düngers.
Löhnis, F.....Handbuch der landwirtschaftlichen Bakteriologie, 1910.
Smith, E. F.....Bacteria in Relation to Plant Diseases, vols. i, 1905; ii, 1911; iii, 1914.

D. *Manuals of Bacteriologic Technic:*

American Public Health Association.....Standard Methods for the Examination of Water and Sewage, 1915.
Burgess, P. S.....Soil Bacteriology Laboratory Manual, 1914.
Eyre, J. W. H.....Bacteriological Technique, 2d ed., 1913.
Giltner, Ward.....Laboratory Manual in General Microbiology, 1916.
Heinemann, P. G.....A Laboratory Guide in Bacteriology, 3d ed., 1915.
Küster, E.....Kultur der Mikroorganismen, 1913.
Löhnis, F.....Landwirtschaftlich-bakteriologisches Praktikum, 1911.
Moore and Fitch.....Bacteriology and Diagnosis, 1914.
Muir and Ritchie.....Manual of Bacteriology, 6th ed., 1913.
Reed, H. S.....A Manual of Bacteriology, 1914.

E. *Classification of Bacteria:*

Chester, F. D.....A Manual of Determinative Bacteriology, 1901.
Lehmann und Neumann...Bakteriologie und bakteriologische Diagnostik., Teil I, Atlas, 1910; Teil II, Text. 1912.
Migula, W.....System der Bakterien, Bd. I, 1897; Bd. II, 1900.
Winslow and Winslow.....Systematic Relationships of the Coccaceæ, 1908.

F. *Journals of Bacteriology and Related Subjects:*

Annales de l'Institute Pasteur, T. I., 1887.
Arb. Biol. Abt. Landw.-und Forstw. K. Gsndhtsamt., Bd. I, 1900.
Biedermann's Centralblatt für Agrikulturchemie, Bd. I, 1872.
Botanical Gazette, vol. i, 1876.
Centralblatt für Bakteriologie, Abt. I, Orignale, Bd. 1, 1887.
Centralblatt für Bakteriologie, Abt. I, Referate, Bd. 31, 1902.
Centralblatt für Bakteriologie (etc.), Abt. 2, Bd. 1, 1892.
Comptes Rendus Académie des Sciences, T., 1835.
Folia Mikrobiologica, 1, 1912.
Jahresbericht über Fortschritte, Gärungs Organismen, Bd. 1, 1890.
Jahresbericht über die Fortschritte der Agrikulturchemie, Bd. 1, 1858.

Jahresbericht über die Landwirtschaft, Bd. 1, 1886.
Journal of Agricultural Science, vol. i, 1906.
Journal of American Society of Agronomy, vol. i, 1910.
Journal of Bacteriology, vol. i, 1916.
Journal of Biological Chemistry, vol. i, 1906.
Journal of Industrial and Engineering Chemistry, vol. i, 1909.
Journal of Infectious Diseases, vol. i, 1904.
Journal für Landwirtschaft, Bd. 1, 1853.
Landwirtschaftliche Jahrbücher, Bd. 1, 1872.
Landwirtschaftliches Jahrbuch der Schweiz, Bd. 1, 1887.
Landwirtschaftliche Versuchs-Stationen, Bd. 1, 1859.
Phytopathology, vol. i, 1911.
Soil Science, vol. i, 1916.
Zeitsch. für Gärungs-Physiologie, Bd. 1, 1912.
Zeitschrift für das landw. Versuchswesen in Oesterreich, Bd. 1, 1898.

Agricultural Index, vol. i, 1916.
Experiment Station Record, vol. i, 1888.
International Catalogue of Scientific Literature, 1901.
Journal of Agricultural Research, vol. i, 1913.
State Experiment Station Bulletins.
United States Department of Agriculture Bulletins.

LABORATORY RULES

Read Carefully the Following Rules:

1. Before pouring plates or making transfers, wash off the desk with a 1 : 1000 mercuric chlorid solution. Hold the test-tube cultures to be transferred as nearly in a horizontal position as possible. Avoid opening cultures in a current of air.

2. All cultures are to be grown in the incubator at 28° C. unless otherwise stated.

3. After using balances, always return weights to their proper places. Do not leave any dust or dirt on balances.

4. All solid material, as soil, agar, cotton or filter-paper, must be emptied into waste jars and not into the sinks.

5. Soil should not be sieved in the laboratory. The greenhouse or potting room may be used for this purpose.

6. At the end of the laboratory period return all stock bottles and chemicals to their proper places on the shelves. See that all apparatus is replaced in the lockers and that all gas-burners are shut off. Wipe off the table top before leaving.

7. Anything left on the desk will be collected after the laboratory period and returned to the store-room.

EXERCISES IN SOIL BACTERIOLOGY

SECTION I

NUMBER OF MICROÖRGANISMS IN SOIL

Directions for Drawing Soil Samples

WHEN it is necessary to secure accurate samples, dig a ditch to the desired depth. By means of a sterile trowel representative samples may be drawn from the sides of the ditch. In this way outside contamination is largely prevented.

Samples from the surface to 1 foot deep may be taken as follows: Remove the coarse surface débris and sink a large, sterile test-tube or metal cylinder to the desired depth. Samples of surface soil may be taken with a sterile spatula. Draw several samples and empty into sterilized paper bags or other vessels. Mix and pulverize the sample. This may be done with a sterile spatula upon a large piece of sterile paper. From the well-mixed sample remove a representative portion for dilution, and at the same time make a moisture determination. As soon as possible after samples are drawn arrange to count.

Exercise 1

Number of Bacteria According to the Dilution Method

1. Add 50 grams of soil to 500 c.c. of sterile water or 500 c.c. of physiologic salt solution.

2. Shake the suspension vigorously for five minutes.

3. Allow the coarse particles to settle and dilute in the following manner:

(*a*) Add 1 c.c. of the soil extract to 99 c.c. of sterile water; equal to 1 : 1000.

(*b*) Add 1 c.c. of dilution (*a*) to 99 c.c. of sterile water; equal to 1 : 100,000.

(*c*) Add 10 c.c. of dilution (*b*) to 90 c.c. of sterile water; equal to 1 : 1,000,000.

(*d*) Add 10 c.c. of dilution (*c*) to 90 c.c. of sterile water; equal to 1 : 10,000,000.

(*e*) Add 10 c.c. of dilution (*d*) to 90 c.c. of sterile water; equal to 1 : 100,000,000.

4. Shake thoroughly between each dilution.

5. Inoculate three tubes of bouillon with 1 c.c. from each dilution.

6. Incubate these at 28° C. for one week. At the end of two-day periods examine the tubes for evidence of growth, as shown by turbidity, pellicles, or sediment. If all cultures in dilutions from (*a*) to (*d*) show growth, there must be 10,000,000 or more bacteria present in 1 gram of soil.

Note.—If it is desired to determine the numbers of specific types of bacteria present, prepare additional liquid media. Use urea bouillon for urea fermenters, peptone solution for ammonifiers, etc. In this way it is possible to secure an approximate idea of the number of the various groups of organisms present in a sample of soil.

Exercise 2

Number of Protozoa According to the Dilution Method

1. Add 50 grams of soil to 500 c.c. of sterile water, as given in the preceding exercise.

2. After the coarse particles have settled, dilute as follows:

(*a*) Add 1 c.c. of the soil suspension to 9 c.c. of sterile water; equal to 1 : 100.
(*b*) Add 1 c.c. of dilution (*a*) to 9 c.c. of sterile water; equal to 1 : 1000.
(*c*) Add 1 c.c. of dilution (*b*) to 9 c.c. of sterile water; equal to 1 : 10,000.
(*d*) Add 1 c.c. of dilution (*c*) to 9 c.c. of sterile water; equal to 1: 100,000.

3. Inoculate two tubes of protozoa media (hay-soil extract, soil extract, or any medium adapted to protozoa) with 1 c.c. of each of the above dilutions (see p. 97).

4. Incubate the protozoa cultures at room temperature.

5. At regular intervals of two days each make a microscopic examination of the cultures. Since the protozoa are usually larger than bacteria—the 16 mm. two-thirds and 4 mm. one-sixth—objectives will be found desirable.

6. By means of a large-mouthed pipet or loop transfer a small portion of the protozoa culture to a slide and examine. A wet or hanging-drop mount may be used. In certain cases the small flagellates become so numerous that it is difficult to distinguish between the bacteria and protozoa.

Note.—An abundant growth of large protozoa may be obtained if mannit solution (m. 39) is inoculated with a small amount of field soil and the culture incubated for one week or longer at room temperature. A drop of the culture treated with Gram's iodin solution will show the presence of numerous ciliates. In certain cases the ciliates are marked by numerous small, deep golden bodies within their cell vacuoles, apparently Azotobacter cells.

Exercise 3

Number of Bacteria According to Plate Method

In order to reduce the error common to determinations of this character, it is well to use a large sample of soil. All weighings should be made as rapidly as possible to avoid errors due to loss of moisture by evaporation. Bal-

ances sensitive to 10 milligrams are satisfactory for this work. Analytic balances may be used, but are not necessary.

1. Weigh 20 to 30 grams of soil on a piece of sterile paper or scoop, or weigh the entire soil sample, bottle, and contents; then remove about 20 grams with a sterile spatula. Reweigh sampling bottle and contents and record loss in weight. Transfer the soil to a 200-c.c. sterile water blank.

Note.—Two hundred c.c. of water in a 500-c.c. Erlenmeyer flask allows ample space for shaking. Tap-water will be found very satisfactory for soil counts. The water blanks may be sterilized in the autoclave for fifteen minutes at 15 pounds' pressure. For ordinary work, provided blanks are not stored for a long time, thirty minutes in the steamer will be sufficient.

2. Shake this suspension vigorously for five minutes and allow the coarse particles to settle.

3. Add 10 c.c. of this first dilution, equivalent to 1 gram of soil, to a 90-c.c. sterile water blank. One c.c. from this dilution is equal to 0.01 gram of soil.

4. After shaking, add 1 c.c. to a 99-c.c. sterile water blank. (Dilution 1 : 10,000.)

5. Transfer 1 c.c. of the above to a 9-c.c. sterile water blank. As a rule, this dilution, which represents 1 : 100,000 of a gram of soil to each cubic centimeter, is the one from which to pour plates. If the soil is very poor, use a dilution of 1 : 10,000; if very rich, 1 : 1,000,000. The number of dilutions will depend on the type of soil. Garden or well-cultivated soil rich in organic matter requires a higher dilution than poor, sandy soil.

6. Pour plates from the following dilutions in triplicate: 1 : 10,000, 1 : 100,000, and 1 : 1,000,000.

7. Add about 10 c.c. of an agar medium, melted and cooled

to 40° C., to each plate. A blank plate or control should be poured with each series. In case the medium is turbid, heat slowly, allowing the deposit to settle. Use only the clear portion of the medium for pouring plates.

8. Immediately after adding the culture-medium rotate each plate to secure a uniform mixture. Allow agar plates to harden on a level surface.

Fig. 1.—Agar plate showing a common form of spreading colonies found in soil.

9. Agar plates should be inverted and incubated under a moist chamber at 28° C. The time of incubation will depend upon the different culture-media. After five to ten days count the number of colonies on each plate. If the colonies are not too thick, it is well to dot each one with a pen. When the colonies are too thick to count easily, use a hand lens and counting plate.

10. Reduce all results to number of bacteria in 1 gram of soil.

Exercise 4

Comparison of the Number of Bacteria on Different Culture-media

1. Weigh out a representative sample of field soil, about 20 grams, and transfer to a sterile 200-c.c. water blank.

2. Carry through dilutions as given in the previous exercise. In the last dilution use 2 c.c. to 18 c.c. of water instead of 1 c.c. to 9 c.c.

3. At the same time the count is made weigh a sample of soil and determine the moisture.

4. Pour triplicate plates of the following media: Heyden-Nährstoff, sodium asparaginate, soil-extract, casein agars, and soil-extract gelatin. After melting, the gelatin may be cooled to 30° C. before pouring plates.

5. Gelatin should be incubated at a constant temperature, about 20° C., for one week. At regular intervals of two days each remove the plates and count the number of colonies, differentiating between the liquefiers and non-liquefiers. In order to prevent a rapid liquefaction of the gelatin, the peptonizing organisms may be killed by touching them with the point of a silver nitrate pencil.

6. Compare the number of peptonizing colonies on casein agar with the number of liquefiers on gelatin.

Note.—After counting the total number of colonies on casein agar, flood the plates with N/20 lactic acid. After the medium turns white the acid may be poured off. The lactic acid precipitates the casein, which produces an opaque white medium except around the peptonizing colonies, where the casein has been digested. These colonies may be distinguished by the clear zone.

7. The results of the plate counts may be tabulated as follows:

TABLE 1.—*Comparison of the Number of Bacteria on Different Culture-media*

Medium.	Bacteria per plate.				Bacteria in 1 gm. of soil.	
	Total.	Average.	Liquefiers.		Average.	Liquefiers.
			Total.	Average.		Average.
Heyden Nährstoff. do. do.						
Sodium asparaginate. do. do.						
Soil-extract agar. do. do.						
Casein agar. do. do.						
Soil-extract gelatin. do. do.						

Exercise 5

Effect of Depth on Number of Bacteria

1. The samples for this exercise should be drawn from virgin soil well removed from any source of contamination. Save some of this soil for Exercise 8, p. 42. The type of soil will determine to a certain degree the number of organisms at different depths.

2. Divide the sample of soil, taking one portion for plate count, the other for moisture determination. For virgin field soil the following dilutions have been found satisfactory.

3. Take soil samples and plate as follows·

(*a*) Surface soil..... 1 : 100,000.
(*b*) Soil 1 foot deep..... 1 : 10,000 and 1 : 100,000.
(*c*) Soil 2 feet deep..... 1 : 1000 and 1 : 10,000.
(*d*) Soil 4 feet deep..... 1 : 100 and 1 : 1000.

Follow the method given in previous exercises.

4. Pour triplicate plates, using the medium that gave the highest count. (See preceding exercise.)

5. Tabulate results as follows:

TABLE 2.—*Effect of Depth on Number of Bacteria*

Position.	Moisture.	Bacteria per plate.		Bacteria in 1 gm. of dry soil.
	Per cent.	Total..	Average.	Average.
Surface. do. do.				
1 foot. do. do.				
2 feet. do. do.				
4 feet. do. do.				

Exercise 6

Effect of Moisture on Number of Bacteria

1. Weigh out 1 kilogram of *air-dry* field soil and mix thoroughly.

TABLE 3.—*Effect of Moisture on Number of Bacteria*

Time.	Moisture.	Dilution.	Bacteria per plate.		Bacteria in 1 gm. of dry soil.
Days.	Per cent.		Total.	Average.	Average.
7	Air dry.				
	do.				
	do.				
7	15				
	15				
	15				
7	30				
	30				
	30				
7	45				
	45				
	45				
21	Air dry.				
	do.				
	do.				
21	15				
	15				
	15				
21	30				
	30				
	30				
21	45				
	45				
	45				

2. Place 200-gram portions of the air-dry soil into four small glass jars. One pint Mason jars may be used.

3. Adjust the moisture content as follows:

(*a*) Air dry.
(*b*) 15 per cent. moisture.
(*c*) 30 per cent. moisture.
(*d*) 45 per cent. moisture.

4. Incubate the soil samples at room temperature.

5. After intervals of one and three weeks determine the number of bacteria according to the plate method. Pour triplicate plates from the dilution of 1 : 100,000.

6. Arrange results in tabular form (see p. 24).

Exercise 7

Effect of Manures on Number of Bacteria

1. Prepare three tumblers or beakers with 100 grams each of field soil.

2. Arrange as follows:

(*a*) Control.[1]
(*b*) Treat with 1 per cent. of finely chopped green clover.
(*c*) Treat with 1 per cent. of well-rotted stable manure.

3. Since these substances contain great numbers of bacteria, especially the stable manure, plate counts should be made of the manures at the time the soils are treated. For this purpose shake 20-gram portions of the manures with 200 c.c. of sterile water. Dilute as given in the previous exercises. Pour plates from the dilutions 1 : 100,000 and 1 : 1,000,000.

4. After mixing thoroughly the soil and manure in tumblers, raise the moisture to two-thirds saturation.

[1] Control or blank is equivalent to no treatment.

5. Cover the soils with Petri dishes and incubate at room temperature.

6. Determine the number of bacteria after one and three weeks.

7. Before drawing the sample for counts mix the contents of the tumblers thoroughly. This may be done with a sterile spatula. In the case of treated soils plate from the dilutions 1 : 100,000 and 1 : 1,000,000.

8. Tabulate results.

TABLE 4.—*Effect of Manures on Number of Bacteria*

Time.	Treatment.	Dilution.	Bacteria per plate.		Bacteria in 1 gm. of dry soil.
Days.	Per cent.		Total.	Average.	Average.
7	None.				
	do.				
	do.				
7	1 clover.				
	do.				
	do.				
7	1 manure.				
	do.				
	do.				
21	None.				
	do.				
	do.				
21	1 clover.				
	do.				
	do.				
21	1 manure.				
	do.				
	do.				

Exercise 8

Effect of Limestone on Number of Bacteria

1. Fill three tumblers with 100 grams each of field soil.
2. Arrange as follows:

(*a*) Control.
(*b*) Treat with 0.5 per cent. of calcium carbonate or one-half enough calcium carbonate to neutralize the soil acidity.
(*c*) Treat with 1 per cent. of calcium carbonate or enough calcium carbonate to neutralize all soil acidity.

TABLE 5.—*Effect of Limestone on Number of Bacteria*

Time.	Treatment.	Dilution.	Bacteria per plate.		Bacteria in 1 gm. of dry soil.
Days.	Per cent.		Total.	Average.	Total.
7	None.				
	do.				
	do.				
7	½ lime.				
	do.				
	do.				
7	1 lime.				
	do.				
	do.				
21	None.				
	do.				
	do.				
21	½ lime.				
	do.				
	do.				
21	1 lime.				
	do.				
	do.				

3. Use a clean spatula to mix the soil and limestone.

4. Add water to bring the moisture to two-thirds saturation. Cover the tumblers with Petri dishes.

5. Allow the soils to incubate at room temperature.

6. Make plate counts after one and three weeks.

7. Arrange the results in a table (see p. 27).

Exercise 9

Effect of Partial Sterilization on Number of Bacteria

1. Weigh out three portions, 200 grams each, of garden or field soil into small jars (pint Mason). The field soil should be mixed thoroughly before sampling.

2. Treat the jars of soil as follows:

(*a*) Control.
(*b*) Heat to 100° C. for one hour.
(*c*) Treat the soil with 1 per cent. of carbon bisulphid. Pour the CS_2 into small holes (use a glass rod) in the soil and cover immediately.

3. After the soil in jar (*c*) has been exposed to the action of carbon bisulphid for one hour, and the soil in (*b*) heated for one hour, determine the number of bacteria in all three soils. In the case of the untreated soil, plate from the dilution 1 : 100,000, the heated soil dilution 1 : 100, and the carbon bisulphid soil dilution 1 : 10,000.

4. The carbon bisulphid jar should be left uncovered for one day.

5. Incubate all of the samples at room temperature.

6. The second plate count should be made *one week* after treatment. Here again, untreated soil, use the dilution 1 : 100,000, and the treated series, dilutions 1 : 100,000 and 1 : 1,000,000.

7. A third count should be made two weeks after treat-

ment, plating from the same dilutions as in the second count.

8. Tabulate the data.

TABLE 6.—*Effect of Partial Sterilization on Number of Bacteria*

Time.	Treatment.	Dilution.	Bacteria per plate.		Bacteria in 1 gm. of dry soil.
			1 2 3	Average.	Average.
Days.					
Beg.	None.				
Beg.	100° C.				
Beg.	1% CS_2				
7	None.				
7	100° C.				
7	1% CS_2				
14	None.				
14	100° C.				
14	1% CS_2				

Exercise 10

Effect of Plant Roots on Number of Bacteria

1. Collect soil samples from the immediate vicinity of the roots of various plants (alfalfa, clover, etc.), and similar samples 1 or 2 feet away from the plants.

2. Prepare plate counts and moisture determinations of these soils.

3. Tabulate results as follows:

TABLE 7.—*Effect of Plant Roots on Number of Bacteria*

No.	Position.	Bacteria per plate.		Bacteria in 1 gm. of dry soil.
		1 2 3	Average.	Average.
1	Near roots.			
2	Away from roots.			

Increase or decrease.....

Exercise 11

Effect of Season on Number of Bacteria

1. In order to study the relation of bacteria to season, select an isolated plot of soil high enough to prevent drainage from above.

2. Draw samples of soil from this plot at intervals of two weeks each during the fall and winter. If it is not possible to make counts so often, plan to take samples when there is a decided change in temperature. If the soil is frozen, it will be necessary to use a pick or hatchet in securing samples.

TABLE 8.—*Effect of Season on Number of Bacteria*

No.	Date.	Moisture.	Temperature of—		Bacteria per plate.		Bacteria in 1 gm. of dry soil.
			Air.	Soil.			
		Per cent.	° C.	° C.	1 2 3	Average.	Average.

3. Prepare plates immediately on arrival at laboratory.

4. At the same time plates are poured make a moisture determination.

5. Record the outside temperature and soil temperature at the time sample is drawn.

6. Tabulate results (see p. 30).

Exercise 12

Effect of Cultivation on Number of Bacteria

1. Prepare two tumblers with 100 grams each of clay soil.

2. Arrange as follows:

(*a*) Untreated.
(*b*) Cultivated every day by stirring with a sterile spatula.

3. Add water to two-thirds saturation. Cover with Petri dishes and keep in the incubator at 28° C.

4. Count the number of bacteria after one, two, and three weeks, pouring plates from the dilution 1 : 100,000.

Exercise 13

Occurrence of Thermophilic Bacteria

1. Incubate several samples of soil and some fresh stable manure at 60° C.

2. In order to prevent evaporation all samples must be kept in a moist chamber. A large glass beaker or metal container may be used. Avoid glass bell jars, since the high temperature may cause them to crack.

3. After one week in the incubator prepare agar plates from the different samples. Pour from dilutions 1 : 1000 and 1 : 10,000.

4. The plates must be incubated at 60° C.

5. Determine the number of thermophilic bacteria in 1 gram of soil.

6. If desirable, a study may be carried on of the bacteria growing at low temperatures.

Exercise 14

Catalytic Power of Soils

1. Arrange three large, heavy walled glass tubes with 5 grams of soil in each.

2. Treat as follows:

(*a*) Fresh soil, no treatment.
(*b*) Heat in the steamer for thirty minutes.
(*c*) Heat in the autoclave at 15 pounds' pressure for fifteen minutes.

3. Set up the following apparatus (Fig. 2): Insert a two-holed rubber stopper into a large test-tube. In one hole fit a piece of short, straight glass tubing closed at one end with a rubber tube and clamp; in the other, a right angle tube of glass connected with a rubber tube 8 to 10 inches long. This rubber tubing should be fitted to a tube of glass bent as shown in Fig. 2 and connected to the gas-measuring tube.

4. Fill the 100-c.c. gas-measuring tube with water and invert mouth under water. Clamp the tube in place.

5. Then add 10 c.c. of a 1.5 per cent solution of hydrogen

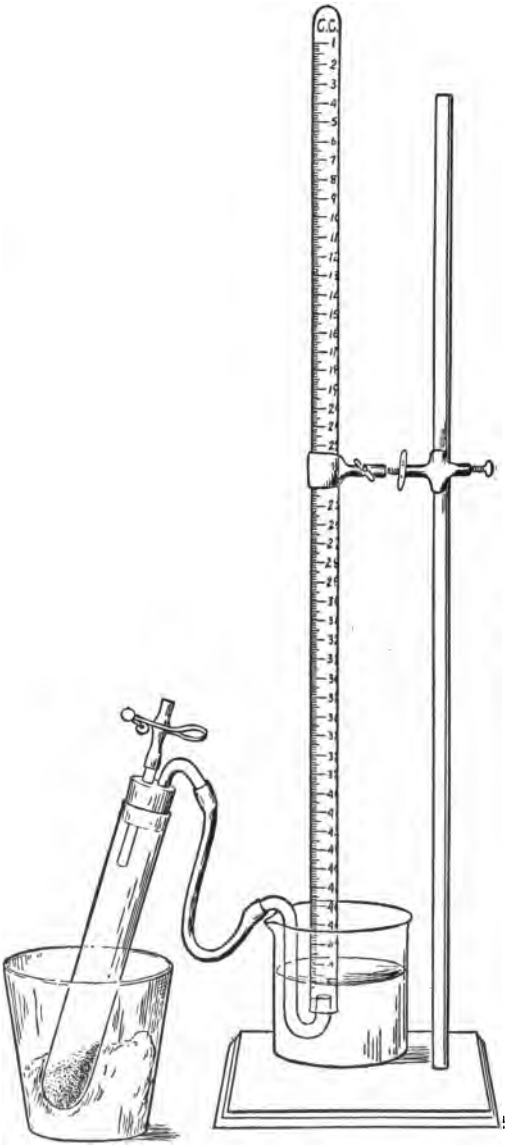


Fig. 2.—Apparatus for determining catalytic power of soils.

peroxid to the soil in large test-tube. Shake the tube at regular intervals.

Note.—For this test 3 per cent. hydrogen peroxid should be made neutral or faintly alkaline to phenolphthalein with dilute sodium hydroxid and diluted to 1.5 per cent.

6. Record the volume of oxygen evolved from a definite quantity of peroxid after ten, thirty, and sixty minutes.

Sullivan, M. X., and Reid, F. R., Bul. 86, U. S. Dept. Agr., Bur. Soils, 1912.

Löhnis, F., Landwirtschaftlich-bakteriologisches Praktikum, p. 120, 1911.

SECTION II

RELATION OF MICROÖRGANISMS TO THE NITROGEN CYCLE

Exercise 1

Ammonification of Urea

1. Prepare six 200-c.c. Erlenmeyer flasks with 50 c.c. each of urea solution (m. 19).

2. After sterilizing, arrange as follows:

(*a*) 1 and 2, control.
(*b*) 3 and 4, inoculate with 1 gm. of soil.
(*c*) 5 and 6, inoculate with 1 gm. of fresh manure.

It is not necessary to weigh accurately the soil or manure.

3. Incubate the cultures at 28° C.

4. After two days remove from each flask 5-c.c. portions of the solution, with a sterile pipet, to a 500-c.c. Erlenmeyer flask.

5. Add about 50 c.c. of distilled water to the urea solution in the large flask, a few drops of methyl red or cochineal, and titrate against N/14 sulphuric acid.

6. From the results of the titrations calculate the amount of ammonia nitrogen in 100 c.c. of the different urea solutions. In order to find the amount of ammonia formed by bacterial action, subtract the untreated from the treated series.

7. Determine the percentage of urea transformed into ammonia.

8. Similar samples may be drawn after three or four days and the amount of ammonia titrated.

9. Tabulate the results.

TABLE 9.—*Ammonification of Urea.*

No.	Inoculum.	Ammonia nitrogen in 100 c.c. of solution.			
		After two days.		After three days.	
		Total.	Per cent.	Total.	Per cent.
1	None.				
2	do.				
3	Soil.				
4	do.				
5	Manure.				
6	do.				

Exercise 2

Isolation of Urea-fermenting Organisms

1. Inoculate duplicate tubes of urea solutions as follows:

(*a*) Medium 19 soil.
(*b*) Medium 19 manure.
(*c*) Medium 20 soil.
(*d*) Medium 20 manure.

A small inoculum is sufficient. In place of tubes (*a*) and (*b*), cultures from the preceding exercise may be used.

2. Two or three days after inoculation examine the test-tube cultures for ammonia production (Nessler's reagent, p. 135).

3. From one tube of each medium showing abundant growth make transfers into tubes of sterile urea solution or water blanks. A wide range of dilutions should be made.

4. Pour plates from the different dilutions with the same medium as in the tube culture plus gelatin (m. 23).

5. Incubate the gelatin plates at 20° C.

6. Examine the plates every forty-eight hours for a period of ten days. The urea organisms are often characterized by a distinct halo around the colonies. Under the low power of the microscope the halo is composed of dumb-bell-shaped crystals.

7. Make transfers to tubes of urea gelatin and incubate for two days.

8. Now test the ammonia-producing power of the pure cultures by inserting sterilized strips of Nessler's paper or turmeric paper in the upper part of the tube.

9. Prepare a stained mount of these two organisms.

Exercise 3

Preparation of Urease From Urea Bacteria

1. Inoculate two large Erlenmeyer flasks, 1000-c.c. capacity, containing 100 c.c. each of urea bouillon (m. 19) with a pure culture of urea fermenter. A very active culture should be used.

2. Incubate at 30° C. until ammonia formation is very evident.

3. In order to secure the urease free of bacteria, filter aseptically through a Berkefeld filter.

4. Prepare a 10 per cent. solution of urea in distilled water. Now mix equal volumes of the filtrate and the urea-water solution.

5. Incubate the enzyme culture at 48° to 50° C. until a large part of the urea nitrogen is converted into ammonia.

6. The action of the urease may be measured by Nesslerizing aliquot portions of the treated and untreated urea-water.

Exercise 4

Ammonification of Gelatin in Solution

1. Prepare eight large Erlenmeyer flasks with 100 c.c. each of gelatin solution (m. 17).

2. Stopper loosely with cotton and sterilize in the autoclave at 15 pounds' pressure for ten minutes.

3. Inoculate six of the flasks with 5 c.c. of a soil suspension.

Note.—Shake 100 gm. of field soil with 200 c.c. of sterile water and allow the coarse particles to settle.

The two remaining flasks keep as controls.

4. Incubate all of the flasks at 28° C.

5. At intervals of two, four, and six days remove duplicate flasks from the incubator and analyze the contents for ammonia (see p. 141). At the time of the last analysis determine the ammonia in controls and subtract from the total amount in the cultures.

6. Tabulate results.

TABLE 10.—*Ammonification of Gelatin in Solution*

No.	Time.	Ammonia nitrogen in 100 c.c. of solution.		Average.	Nitrogen ammonified.
		Total.	Blank subtracted.		
	Days.	Mgm.	Mgm.	Mgm.	Per cent.
1	2				
2	2				
3	4				
4	4				
5	7				
6	7				

Exercise 5

Isolation of Ammonifying Organisms

1. As soon as the cultures of the preceding exercise begin to show a vigorous ammonia production, make loop transfers to tubes of sterile gelatin solution or sterile water.

2. Repeat the dilution four times.

3. Pour gelatin (m. 2) or agar (m. 3) plates from dilutions 1 : 10,000 and 1 : 1,000,000, using 1-c.c. portions for each plate.

4. After colonies have developed, pick several pure cultures. These should be transferred to gelatin solution and their ammonifying power studied. For this purpose use Nessler's reagent.

5. Select two of the organisms that produce the largest amount of ammonia. These should be kept for a later study.

6. Prepare stained mounts of these organisms.

Exercise 6

Ammonification of Various Substances

1. Prepare twelve 50-gram portions of field-soil in clean, dry tumblers. The soil should be mixed thoroughly before samples are drawn.

2. Treat the soils as follows:

(*a*) 1, 2, 3, and 4, add 1 per cent. of casein.
(*b*) 5, 6, 7, and 8, add 1 per cent. of blood-meal.
(*c*) 9, 10, 11, and 12, add 1 per cent. of dried clover hay.

3. Add these substances in the powdered form and mix thoroughly.

4. Bring the moisture content of the soil to two-thirds saturation.

Note.—In order to secure the proper moisture content it is necessary to take into account the water-holding capacity of the added substances, *e. g.*, 1 gram of casein or blood-meal requires about 2 c.c. of water; 1 gram of clover, about 5 c.c. of water.

Allow the soil to stand for one hour or more and remix with a sterile spatula.

5. Determine the amount of ammonia nitrogen in samples 1, 2, 5, 6, 9, and 10 at once. The ammonia nitrogen of these cultures or controls is to be subtracted from the ammonia nitrogen found at the final analysis. This gives the figures "blank substracted."

6. Cover the remaining tumblers with Petri dishes and incubate for four to six days at 28° C. Now determine the ammonia content of all of the soils. The percentage of nitrogen in the different substances is given on the label.

7. Tabulate results.

TABLE 11.—*Ammonification of Various Substances*

No.	Treatment.	Ammonia nitrogen in 100 gm. of soil.		Average.	Nitrogen ammonified.
		Total.	Blank subtracted.		
		Mgm.	Mgm.	Mgm	Per cent.
1	Casein.				
2	do.				
3	Blood-meal.				
4	do.				
5	Clover.				
6	do.				

Exercise 7

Effect of Soil Type on Rate of Ammonification

1. Weigh out four 50-gram portions of garden soil, field soil, and acid soil into clean, dry tumblers.

2. Mix thoroughly 1 per cent. of ground clover or powdered casein with each soil.

3. Determine at once the ammonia nitrogen in duplicate tumblers of the various soils.

4. Add water to the soil of the six remaining tumblers until it is two-thirds saturated. After standing for one hour stir with a spatula.

5. Cover with Petri dishes and incubate for six days or longer. If casein is used, the time of incubation may be shortened to two or three days. In the case of clover no definite date need be followed, one week or longer will not seriously change the results.

6. Determine the amount of ammonia nitrogen.

7. Arrange the data in a table.

TABLE 12.—*Effect of Soil Type on Rate of Ammonification*

No.	Soil type.	Ammonia nitrogen in 100 gm. of soil.		Average.	Nitrogen ammonified.
		Total.	Blank subtracted.		
		Mgm.	Mgm.	Mgm.	Per cent.
1					
2					
3					
4					
5					
6					

Exercise 8

Effect of Depth on Rate of Ammonification

1. A portion of the soil from Exercise 5, page 22, may be used for this study.

2. Prepare sixteen tumblers with 50 grams of soil in each tumbler.

3. In order to prevent contamination of the soil the tumblers and clover must be sterilized.

4. Arrange thus:

(*a*) 1 and 2, surface soil.
3 and 4, surface soil plus 1 per cent. clover.
(*b*) 5 and 6, 1 foot deep.
7 and 8, 1 foot deep plus 1 per cent. clover.
(*c*) 9 and 10, 2 feet deep.
11 and 12, 2 feet deep plus 1 per cent. clover.
(*d*) 13 and 14, 4 feet deep.
15 and 16, 4 feet deep plus 1 per cent. clover.

5. After six days' incubation at 28° C. determine the amount of ammonia.

6. Tabulate.

TABLE 13.—*Effect of Depth on Rate of Ammonification*

No.	Position.	Ammonia nitrogen in 100 gm. of soil.			Nitrogen ammonified.
		Total.	Blank subtracted.	Average.	
		Mgm.	Mgm.	Mgm.	Per cent.
1	Surface.				
2	do.				
3	1 foot.				
4	do.				
5	2 feet.				
6	do.				
7	4 feet.				
8	do.				

Exercise 9

Effect of Moisture on Rate of Ammonification

1. Weigh into tumblers eight portions, 50 grams each, of air-dry field soil.

2. Add 1 per cent. of clover or casein to each tumbler.

3. Arrange thus:

(*a*) 1 and 2, air dry.
(*b*) 3 and 4, 15 per cent. of moisture.
(*c*) 5 and 6, 30 per cent. of moisture.
(*d*) 7 and 8, 45 per cent. of moisture.

Note.—The amount of moisture will depend on the soil type. In this case 45 per cent. represents saturation.

4. Incubate at 28° C. for seven days. If casein is used, four days is long enough.

5. Analyze and compare the results of this test with those of Exercise 6, page 24. Prepare a combination table from the results of these two exercises.

TABLE 14.—*Effect of Moisture on Rate of Ammonification*

No.	Moisture.	Ammonia nitrogen in 100 gm. of soil.			Nitrogen ammonified.
		Total.	Blank subtracted.	Average.	
	Per cent.	Mgm.	Mgm.	Mgm.	Per cent.
1	Air dry.				
2	do.				
3	15				
4	15				
5	30				
6	30				
7	45				
8	45				

Exercise 10

Effect of Limestone on Rate of Ammonification

1. Prepare eight tumblers with 50 grams each of soil. Use two types, a neutral and an acid soil.

2. Add to each soil 1 per cent. of clover or casein.

3. Bring the moisture content up to optimum for ammonification.

4. Arrange as follows:

(*a*) 1 and 2, neutral field soil.
(*b*) 3 and 4, neutral field soil plus 1 per cent. of $CaCO_3$.
(*c*) 5 and 6, acid field soil.
(*d*) 7 and 8, acid field soil plus 1 per cent. of $CaCO_3$.

5. Determine the amount of ammonia after four or six days.

TABLE 15.—*Effect of Limestone on Rate of Ammonification*

No.	Soil type.	Calcium carbonate.	Ammonia nitrogen in 100 gm. of soil.		
			Total.	Average.	Gain or loss from limestone.
		Per cent.	Mgm.	Mgm.	Mgm.
1		None.			
2		do.			
3		1 $CaCO_3$			
4		do.			
5		None.			
6		do.			
7		1 $CaCO_3$			
8		do.			

Exercise 11

Effect of Dibasic Potassium Phosphate on Rate of Ammonification

1. Prepare ten 50-gram portions of soil in tumblers.
2. Add 1 per cent. clover and hold the moisture at two-thirds saturation.
3. Arrange as follows:

(*a*) 1 and 2, analyze at once.
(*b*) 3 and 4, control.
(*c*) 5 and 6, plus 0.1 per cent. K_2HPO_4.
(*d*) 7 and 8, plus 0.2 per cent. K_2HPO_4.
(*e*) 9 and 10, plus 0.5 per cent. K_2HPO_4.

Note.—The dibasic potassium phosphate is readily added from a solution. For this purpose prepare a stock solution.

4. Incubate at room temperature for four days.
5. Determine the amount of ammonia.

TABLE 16.—*Effect of Dibasic Potassium Phosphate on Rate of Ammonification*

No.	Treatment.	Ammonia nitrogen in 100 gm. of soil.			Nitrogen ammonified.
		Total.	Blank subtracted.	Average.	
	Per cent.	Mgm.	Mgm.	Mgm.	Per cent.
1	None.				
2	do.				
3	0.1 K_2HPO_4				
4	do.				
5	0.2 K_2HPO_4				
6	do.				
7	0.5 K_2HPO_4				
8	do.				

Exercise 12

Ammonification by Pure Cultures of Bacteria

1. Prepare ten portions, 50 grams each, of field soil.

2. Add 1 gram of clover and bring the moisture to two-thirds saturation.

3. Cover the tumblers with Petri dishes and sterilize in the autoclave at 15 pounds' pressure for two hours on two consecutive days.

4. When cool, inoculate each tumbler of soil with a 1-c.c. suspension of the bacterial culture to be tested.

Note.—Prepare a suspension of bacteria by shaking a forty-eight-hour-old culture of the organism in sterile water.

(*a*) 1 and 2, uninoculated.
(*b*) 3 and 4, unknown ammonifier (1).
(*c*) 5 and 6, unknown ammonifier (2).
(*d*) 7 and 8, Bacillus subtilis.
(*e*) 9 and 10, Bacillus tumescens.

5. Numbers 1 and 2 should be analyzed at once.

6. Incubate for ten days at 28° C.

7. At the end of this time analyze.

TABLE 17.—*Ammonification by Pure Cultures of Bacteria*

No.	Culture.	Ammonia nitrogen in 100 gm. of soil.			Nitrogen ammonified.
		Total.	Blank subtracted.	Average.	
		Mgm.	Mgm.	Mgm.	Per cent.
1	No. 1				
2	do.				
3	No. 2				
4	do.				
5	B. subtilis				
6	do.				
7	B. tumescens				
8	do.				

Exercise 13

Nitrification in Solution

A. *Nitrite Qualitative:*

1. Prepare five 150-c.c. Erlenmeyer flasks with 20-c.c. portions each of nitrite solution (m. 26).

2. Inoculate two of the flasks.

(*a*) Add approximately 0.1 gram of field soil.
(*b*) Add approximately 0.1 gram of garden soil.

3. Incubate at 28° C.

4. At regular intervals of four to six days remove, with a sterilized platinum needle, 1 drop of the solution from each flask and test as follows:

(*a*) Presence of nitrites—Trommsdorf's reagent (see page 136).
(*b*) Absence of ammonia—Nessler's reagent (see page 135).

Use the spot plate for this test. Record the date and results of test in the table on p. 48.

5. As soon as the culture shows the presence of nitrites and absence of ammonia, make loop subinoculations into a sterile flask of the same medium.

6. If it is desirable to study the nitrite bacteria in enrichment cultures, repeated subinoculations may be made.

B. *Nitrate Qualitative:*

1. Prepare five 150-c.c. Erlenmeyer flasks with 20-c.c. portions each of nitrate solution (m. 28).

2. Inoculate two of the flasks.

(*a*) Add approximately 0.1 gram of field soil.
(*b*) Add approximately 0.1 gram of garden soil.

TABLE 18.—*Nitrification in Solution*

Time in days.	Nitrites.									Nitrates.								
	Field soil.			Garden soil.			Control.			Field soil.			Garden soil.			Control.		
	NH_3	N_2O_3	N_2O_5	NH_3	N_2O_3	N_2O_5	NH_3	N_2O_3	N_2O_3	NH_3	N_2O_3	N_2O_5	NH_3	N_2O_3	N_2O_5	NH_3	N_2O_3	N_2O_5

3. Incubate at 28° C.

4. At regular intervals of four and six weeks remove, with a sterilized platinum needle, 1 drop of the solution from each flask and test as follows:

(*a*) Absence of nitrites—Trommsdorf's reagent (see page 136).
(*b*) Presence of nitrates—Diphenylamin reagent (see page 137).

Use the spot plate for this test. Record the date and results of tests in the table below.

5. As soon as the culture shows the presence of nitrates and absence of nitrites, make loop subinoculations into a sterile flask of the same medium.

6. If it is desirable to study the nitrate bacteria in enrichment cultures, repeated subinoculations may be made.

C. *Nitrification Quantitative:*

1. Place 100-c.c. portions of medium 29 into eight 1-liter Erlenmeyer flasks.

2. Inoculate each flask with 1-c.c. portions of water extract of different soils.

Note.—Shake 50 grams of soil with 100 c.c. of sterile water and allow to settle.

(*a*) 1, 2, 3, and 4, field soil.
(*b*) 5, 6, 7, and 8, garden soil.

3. Immediately after inoculation add 5 c.c. of concentrated sulphuric acid to flasks numbered 1, 2, 5, and 6.

4. Incubate all of the flasks at 28° C. for three to four weeks.

5. At the end of this time determine the amount of nitrate nitrogen (see page 143).

6. Tabulate results as shown on p. 50.

TABLE 19.—*Nitrification in Solution (Quantitative).*

No.	Inoculum.	Nitrate nitrogen in 100 c.c. of solution.			Nitrified.
		Total.	Blank subtracted.	Average.	
		Mgm.	Mgm.	Mgm.	Per cent.
1	Field soil.				
2	do.				
3	Garden soil.				
4	do.				

Exercise 14

Isolation of Nitrifying Organisms

1. Prepare eight tubes of acid sodium potassium silicate (m. 30).

2. Dilute the second enrichment cultures, nitrite, and nitrate organisms until 1 c.c. represents 1 : 1000, 1 : 10,000, 1 : 100,000, and 1 : 1,000,000 of the original culture.

3. Pour the acid silicate into a sterile Petri dish with the culture dilutions; add the nutrient salts and enough sodium carbonate to harden the silicate.

4. When hard, invert the plates and incubate under a moist bell jar for three to six weeks.

5. Examine at weekly intervals, using the low-power objective. As soon as small colonies appear, make transfers to sterile nitrite or nitrate solution.

6. The nitrifying organisms may be grown on washed agar (m. 31).

Exercise 15

Nitrification of Various Substances

1. Prepare six portions of field soil, 100 grams each, in tumblers or flasks. Mix and sieve the soil well before using.

2. Treat as follows:

(*a*) 1 and 2, untreated.
(*b*) 3 and 4, 30 mgm. of nitrogen from $(NH_4)_2SO_4$.
(*c*) 5 and 6, 30 mgm. of nitrogen from casein.

The proper amount of nitrogen is most conveniently added from solution. Prepare a stock solution in such a way that 5 c.c. equals 30 milligrams of nitrogen in the form of casein or ammonium sulphate.

3. Mix these substances thoroughly with the soil.

4. Add sterile water to make half-saturation.

5. Cover with Petri dishes and incubate at 28° C.

6. Weigh each week and restore loss of water by evaporation.

7. Analyze for nitrate nitrogen after ten and twenty days.

8. Express the results in terms of milligrams of nitrate nitrogen in 100 grams of soil; also as percentages of the original substance nitrified.

9. Tabulate results as follows:

TABLE 20.—*Nitrification of Various Substances*

No.	Treatment.	Nitrate nitrogen in 100 gm. of soil.				Nitrified.
		After ten days.		After twenty days.		
		Total.	Blank subtracted.	Total.	Blank subtracted.	
	Mgm.	Mgm.	Mgm.	Mgm.	Mgm.	Per cent.
1	None.					
2	30 N. from $(NH_4)_2SO_4$					
3	30 N. from casein.					

Exercise 16

Effect of Soil Type on Nitrification

1. Weigh out four 100-gram portions of garden soil, field soil, and acid soil into clean, dry tumblers.

2. In one-half of the tumblers mix thoroughly with the soil 1 per cent. of clover tissue.

3. Arrange as follows:

(*a*) 1 and 2, garden soil untreated.
(*b*) 3 and 4, garden soil plus 1 per cent. clover.
(*c*) 5 and 6, field soil untreated.
(*d*) 7 and 8, field soil plus 1 per cent. clover.
(*e*) 9 and 10, acid soil untreated.
(*f*) 11 and 12, acid soil plus 1 per cent. clover.

4. Add moisture until the soil is two-thirds saturated, allow it to stand for one hour, and remix.

5. Cover with Petri dishes and incubate at 28° C.

6. After three weeks determine the nitrate nitrogen (see p. 143) in numbers 1, 3, 5, 7, 9, and 11; after 6 weeks in numbers 2, 4, 6, 8, 10, and 12.

TABLE 21.—*Effect of Soil Type on Nitrification*

No.	Soil and treatment.	Nitrate nitrogen in 100 gm. of soil.				Nitrified.
		After twenty-one days.		After forty-two days.		
		Total.	Blank subtracted.	Total.	Blank subtracted.	
	Per cent.	Mgm.	Mgm.	Mgm	Mgm.	Per cent.
1	Garden.					
2	Garden 1 clover.					
3	Field.					
4	Field 1 clover.					
5	Acid.					
6	Acid 1 clover.					

Exercise 17

Effect of Moisture on Nitrification

1. Prepare eight 100-gram portions of field soil in clean tumblers.

2. Add to each, 30 milligrams of nitrogen in the form of ammonium sulphate.

3. Arrange thus:

(*a*) 1 and 2, air dry.
(*b*) 3 and 4, 15 per cent. moisture.
(*c*) 5 and 6, 30 per cent. moisture.
(*d*) 7 and 8, 45 per cent. moisture.

4. After standing for one hour, mix thoroughly the contents of the tumblers.

5. Cover with Petri dishes and incubate for fourteen days at 28° C.

6. At the end of the period determine the nitrate nitrogen.

7. Tabulate results.

TABLE 22.—*Effect of Moisture on Nitrification*

No.	Moisture.	Nitrate nitrogen in 100 gm. of soil.			Nitrified.
		Total.	Blank subtracted.	Average.	
	Per cent.	Mgm.	Mgm.	Mgm.	Per cent.
1	Air dry.				
2	do.				
3	15				
4	15				
5	30				
6	30				
7	45				
8	45				

Exercise 18

Effect of Limestone on Nitrification

1. Prepare ten tumblers of soil, 100 grams in each. Two types of soil may be used, neutral and acid.

2. Arrange each soil type as follows:

(*a*) 1 and 2, untreated.
(*b*) 3 and 4, 30 mgm. of nitrogen as ammonium sulphate.
(*c*) 5 and 6, 30 mgm. of nitrogen as ammonium sulphate plus 1 gram $CaCO_3$.
(*d*) 7 and 8, 30 mgm. of nitrogen as gelatin.
(*e*) 9 and 10, 30 mgm. of nitrogen as gelatin plus 1 gram $CaCO_3$.

3. Stir in the chemicals thoroughly by means of a sterile spatula.

4. Add water and incubate for ten to twenty days at room temperature. From time to time replace the water lost by evaporation.

5. At the end of the period of incubation analyze for nitrates.

TABLE 23.—*Effect of Limestone on Nitrification*

No.	Treatment.	Nitrate nitrogen in 100 gm. of soil.			Nitrified.
		Total.	Blank subtracted.	Average.	
	Per cent.	Mgm.	Mgm.	Mgm.	Per cent.
1	None.				
2	do.				
3	Ammonium sulphate.				
4	do.				
5	Ammonium sulphate plus 1 limestone.				
6	do.				
7	Gelatin.				
8	do.				
9	Gelatin plus 1 limestone.				
10	do.				

Exercise 19

Isolation of Denitrifying Organisms

1. Fill five test-tubes about two-thirds full of denitrifying solution (m. 33).

2. Inoculate as follows:

(*a*) Uninoculated.
(*b*) Inoculated with approximately 0.1 gram of garden soil.
(*c*) Inoculated with approximately 0.1 gram of fresh manure.

3. Incubate at 28° C. until all nitrates have disappeared. The destruction of nitrates is generally indicated by foaming.

4. At regular intervals, daily if possible, make qualitative tests (spot plate) for the presence of nitrates, nitrites, and ammonia.

5. As soon as the nitrates are destroyed transfer a loopful of the old culture to a new tube of denitrifying solution. This may be repeated several times, although a pure culture is readily isolated from the second transfer.

6. Follow the same method of isolation as given in the previous exercises. Pour plates of denitrifying agar, and incubate them until the plates show a good growth.

7. Now pick off several isolated colonies, making transfers into tubes of sterile denitrifying solution.

8. From the pure culture showing the most vigorous denitrification make a transfer to denitrifying agar. Preserve this pure culture for later study.

Exercise 20

Reduction of Nitrates to Nitrites

1. Prepare four tubes of starch nitrate agar (m. 35).

2. Dilute two soil types with sterile water until 1 c.c. represents from 10 to 50 organisms.

3. From these suspensions of soil bacteria prepare duplicate plates with starch-nitrate agar.

4. When the colonies are well developed, pour over the surface of *one* of the duplicate plates a very dilute solution of potassium iodid in dilute hydrochloric acid. Allow to react for a moment or two, then pour off. The production of a blue zone around colonies indicates a reduction of nitrates to nitrites.

$$KI + HCl = KCl + HI$$

$$2HI + 2HNO_2 = 2H_2O + 2NO + I_2$$

5. Note the relative proportion of organisms capable of reducing nitrates to nitrites.

6. Note the general characteristics of such colonies, and from similar colonies upon the untreated plates make transfers to nitrate agar slopes (m. 33).

Exercise 21

Reduction of Stains by Denitrifying Organisms

1. Inoculate duplicate tubes of nitrate solution (m. 33) with pure cultures of denitrifying organisms.

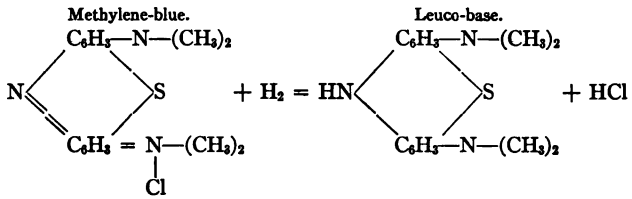
2. Add to each tube 0.5 c.c. of a sterile 1 : 1000 (highest purity) methylene-blue solution and mix thoroughly.

3. In order to exclude partially the oxygen, pour paraffin oil to a depth of about 2 cm. in one-half of the tubes.

4. Incubate at 28° C.

5. Note each day the change in color. This change furnishes a method for detecting nitrites. As long as nitrites are present the solution remains blue. A colorless solution indicates that all of the nitrite nitrogen is destroyed.

This should be confirmed by qualitative tests with Trommsdorf's and diphenylamin reagents.



Exercise 22

Denitrification by Pure Cultures of Bacteria

1. Prepare ten 200-c.c. portions of denitrifying solution (m. 33) in 300-c.c. Erlenmeyer flasks.

2. Inoculate as follows:

(*a*) 1 and 2, uninoculated.
(*b*) 3 and 4, pure culture of unknown denitrifier from Exercise 19.
(*c*) 5 and 6, Bacillus pyocyaneus.
(*d*) 7 and 8, Bacillus Hartlebii.
(*e*) 9 and 10, Bacillus coli.

3. Incubate all cultures for two weeks at 28° C.

4. At the end of the incubation period make qualitative tests of each culture for ammonia, nitrites, and nitrates. If present, determine the amount according to quantitative methods.

5. In all of the cultures, inoculated and uninoculated, determine the total nitrogen. *Use the modified Kjeldahl method* (see page 147). For total nitrogen analysis take duplicate portions of 50 c.c. each of the cultures.

6. For determining the nitrate nitrogen take 10-c.c. portions of the control, dilute with 500 c.c. of distilled water, and of this evaporate 10-c.c. portions to dryness.

In the case of the inoculated cultures with nitrates present proceed as follows: (*a*) Evaporate 10 c.c. to dryness, and (*b*) dilute 10 c.c. to 100 c.c., and evaporate 10 c.c. of this to dryness (see page 143).

7. From the results of the quantitative analyses fill in the following table:

TABLE 24.—*Denitrification by Pure Cultures of Bacteria*

No.	Treatment.	Nitrogen in 100 c.c. of solution.					
		As nitrate.			Total nitrogen.		
		Begin.	End.	Loss.	Begin.	End.	Loss.
		Mgm.	Mgm.	Mgm.	Mgm.	Mgm.	Mgm.
1	Control.						
2	do.						
3	Unknown denitrifier.						
4	do.						
5	Bacillus pyocyaneus.						
6	do.						
7	Bacillus Hartlebii.						
8	do.						
9	Bacillus coli.						
10	do.						

Exercise 23

Denitrification with the Formation of Nitrous Oxid (Optional)

1. Prepare in glass-stoppered bottles medium 34 plus 80 grams potassium nitrate in each liter.

2. Inoculate two bottles with 10 to 20 grams of garden soil; two with a pure culture of a denitrifier.

3. Incubate at 37° C.

4. Place bottles in plates, so that the overflow is collected.

5. After forty-eight to seventy-two hours remove the stopper and insert a glowing splinter. The nitrous oxid should behave much like pure oxygen.

Exercise 24

Denitrification in Soil

1. Prepare eight 100-gram samples of field soil in tumblers.

2. Add to each 100 grams of soil 60 milligrams of nitrogen in the form of potassium nitrate.

3. Treat the series as follows:

(*a*) 1 and 2, control untreated.
(*b*) 3 and 4, add 2.5 grams of dextrose.
(*c*) 5 and 6, control untreated.
(*d*) 7 and 8, add 2.5 grams of dextrose.

4. Mix these materials thoroughly by means of a spatula.

5. To soil portions 1 to 4 add sterile water to bring the moisture content to about one-half saturation.

6. To soil portions 5 to 8 add sterile water to bring moisture up to total saturation.

7. Incubate for two weeks at 28° C.

8. At the end of this time remove a sample for nitrate determination and dry the remainder for total nitrogen analysis. Use the *modified Kjeldahl method to include nitrates* (see page 147).

9. From these results calculate the percentage of the nitrogen denitrified, and note the effect of excessive moisture and excessive organic matter on the loss of nitrogen.

10. Tabulate results.

TABLE 25.—*Denitrification in Soil*

No.	Treatment.	Nitrogen in 100 gm. of soil.					
		As nitrate.			Total nitrogen.		
		Begin.	End.	Loss.	Begin.	End.	Loss.
		Mgm.	Mgm.	Mgm.	Mgm.	Mgm.	Mgm.
1 2	½ water control. do.						
3 4	½ water 2.5 dextrose. do.						
5 6	Total water control. do.						
7 8	Total water 2.5 dextrose do.						

Exercise 25

Autotrophic Denitrifying Bacteria

1. Prepare six tall tubes of Lieske's culture-medium (m. 38). The tubes for this exercise should be at least 10 to 15 inches long and filled four-fifths full of the culture-medium.

2. Inoculate:

(*a*) 1 and 2, uninoculated.
(*b*) 3 and 4, 1 gram of garden soil.
(*c*) 5 and 6, 1 c.c. of sewage.

3. Incubate the cultures for six to eight weeks at 28° C.

4. Determine the total nitrate content of the different cultures.

Exercise 26

Nitrogen Fixation in Solution

1. Measure into four 750-c.c. Erlenmeyer flasks 100-c.c. portions of mannit solution (m. 39).

2. Weigh accurately just 10 grams of soil into each flask.

3. To two of the flasks add concentrated sulphuric acid at once, or sterilize.

4. Incubate the cultures for three weeks at 28° C.

5. At the end of this time analyze for total nitrogen according to the Kjeldahl method (see page 145).

6. Subtract the nitrogen in the soil and culture at the beginning, from that in the culture after three weeks' growth. The difference represents the amount of nitrogen fixed by micro-organisms.

Exercise 27

Isolation of Azotobacter

1. Prepare four flasks (100-c.c. Erlenmeyer) of mannit liquid medium, 20 c.c. in each.

2. Inoculate with 1 or 2 grams of soil.

3. Incubate at 28° C. and note changes occurring in cultures.

4. Every two days examine the films in hanging-drop preparations and note the predominating type of organism. Also examine some of the surface film in a drop of water mixed with a drop of Meissner's or Gram's iodin solution (see pages 127, 128).

5. Dilute two loops of surface film in a 100-c.c. sterile water blank containing 50 grams of clean sand.

Note.—The presence of sand in the water blank aids in breaking up the gelatinous clumps of the Azotobacter.

6. Shake vigorously, and transfer 1 c.c. to a second 100-c.c. blank, and so on to a third.

7. From the third dilution pour plates, using 1 c.c. for each. It is frequently difficult to separate Azotobacter from a small organism known as *Bacillus radiobacter*.

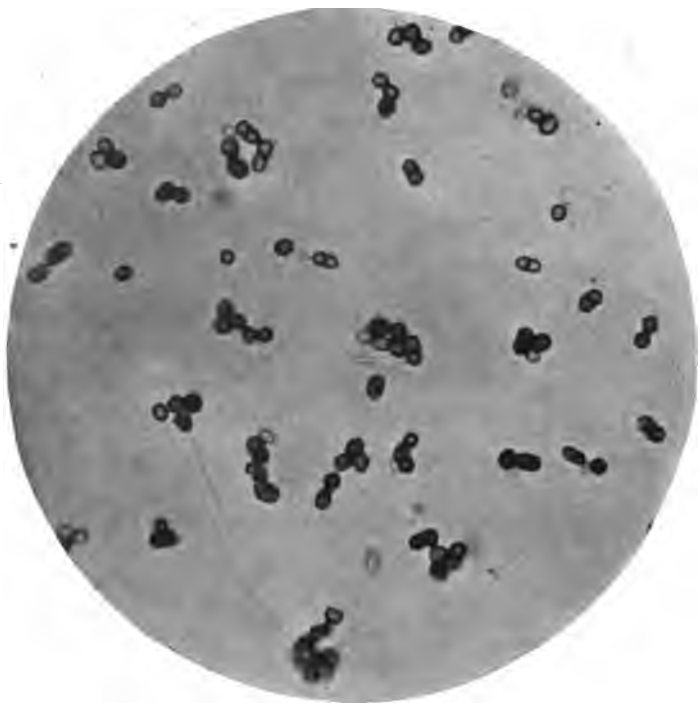


Fig. 3.—Azotobacter stained with methylene-blue; × 1200.

Exercise 28

Nitrogen Fixation in Soil

1. Weigh two 500-gram portions of field soil into soup plates.

2. Treat as follows:

(*a*) Control untreated.
(*b*) Add 2 per cent. of mannit.

3. Mix the mannit thoroughly with the soil by means of a spatula.

4. Raise the moisture content of the soil to optimum, and at regular intervals of two days add water to replace the loss by evaporation.

5. Incubate these at 28° C. for from fourteen to twenty-one days.

6. Now prepare the soil for analysis. When dry, pass it through a 20-mesh sieve, mix thoroughly, and draw a small sample for analysis; about 100 to 150 grams is enough. This smaller sample should be pounded in a mortar until the entire mass passes through a 100-mesh sieve. Weigh out from three to six portions of 10 grams each into 800-c.c. Kjeldahl flasks.

7. Analyze according to the Kjeldahl method (see p. 145).

8. Run moisture determinations on the soil at the time samples are taken for nitrogen analysis.

9. Tabulate results.

TABLE 26.—*Nitrogen Fixation in Soil*

No.	Treatment.	Nitrogen in 100 gm. of dry soil.		
		Total.	Average.	Gain due to treatment.
	Per cent.	Mgm.	Mgm.	Mgm.
1	None.			
2	do.			
3	do.			
4	2 mannit.			
5	do.			
6	do.			

Exercise 29

Nitrogen Fixation by Pure Cultures of Azotobacter

1. Prepare six 1-liter Erlenmeyer flasks with 100 c.c. each of mannit agar. In place of the flasks large pans or moist chambers may be used. The object is to use a vessel that will give a large surface exposure.

2. After sterilization, inoculate the agar films with a pure culture of Azotobacter. This may be accomplished by using 1-c.c. transfers from a suspension in sterile water.

3. Immediately after inoculation remove half of the cultures for analysis. These may be treated with sulphuric acid or sterilized.

4. A few days after inoculation add 10 c.c. of sterile water to each culture.

5. Incubate the cultures in such a position that only a portion of the surface will be covered with water, and from day to day rotate. In this way it is possible to get an even film over the entire surface.

6. About 28° C. is a favorable temperature for growth.

7. After twenty-one days analyze all of the cultures for total nitrogen.

TABLE 27.—*Nitrogen Fixation by Pure Cultures of Azotobacter*

No.	Treatment.	Nitrogen in 100 c.c. of agar.		
		Total.	Average.	Gain.
	Per cent.	Mgm.	Mgm.	Mgm.
1	None.			
2	do.			
3	do.			
4	Azotobacter.			
5	do.			
6	do.			

Exercise 30

Effect of Variation in Culture-media on the Growth of Azotobacter (Optional)

1. Prepare eight tubes of agar slopes.
2. Arrange the culture-media as follows:

(*a*) 1 and 2, mannit agar.
(*b*) 3 and 4, agar without mannit.
(*c*) 5 and 6, agar with lactose in place of mannit.
(*d*) 7 and 8, mannit agar without phosphate.

3. Inoculate all cultures from a suspension of Azotobacter.
4. Incubate at 28° C. Examine every two days for twelve days or longer.
5. Record the growth of Azotobacter on the various culture-media.

Exercise 31

Relation of Azotobacter to Oxygen

1. Prepare six mannit agar slope cultures of Azotobacter chroöcoccum.
2. Treat as follows:

(*a*) 1 and 2, untreated.
(*b*) 3 and 4, place in an atmosphere free of oxygen (see page 133).
(*c*) 5 and 6, seal the tubes by melting the glass and drawing out the ends.

3. Incubate at room temperature.
4. Examine weekly for growth and pigment formation. If the sealed tubes fail to show a brown to black pigment after two weeks, open and note the change in color after three or four days.

Exercise 32

Anaërobic Nitrogen Fixation (Clostridiæ)

1. Prepare four small flasks of Winogradsky's solution (m. 41). Arrange to have the liquid high in the necks of the flasks.

2. After sterilization inoculate the liquid with a pasteurized soil extract.

Note.—Heat 50 grams of soil with 200 c.c. of water for fifteen minutes at 80° C.

3. Allow the coarse particles to settle and pipet 5-c.c. portions into the flasks.

4. Arrange as follows:

(*a*) 1 and 2, sterilize immediately, or add 5 c.c. of sulphuric acid.
(*b*) 3 and 4, incubate at 28° C.

5. After twenty-one days analyze for total nitrogen. Transfer the entire contents to a Kjeldahl flask. In order to avoid too rapid evolution of carbon dioxid, the sulphuric acid should be added slowly.

Exercise 33

Isolation of Anaërobic Nitrogen-fixing Organisms

1. From the cultures obtained in the previous exercise make transfers into tubes of sterile Winogradsky's solution.

2. Incubate under anaërobic conditions for ten to fourteen days at 28° C.

3. Inoculate from these cultures into tubes of sterile water, and from these dilutions into two tubes (in series) of Winogradsky's agar liquefied and cooled to 40° C.

4. Pour plates in the usual way.

5. Incubate under anaërobic conditions for seven to ten days at 20° C., and make transfers from several well-isolated colonies into tubes of Winogradsky's solution, and incubate as before. If the cultures are not pure, they should be replated.

Exercise 34

Isolation of Bacillus Radicicola from Different Legumes

1. Thoroughly wash the roots of several legumes (*e. g.*, red clover, alfalfa, sweet clover, vetch, and soy beans) under the tap.

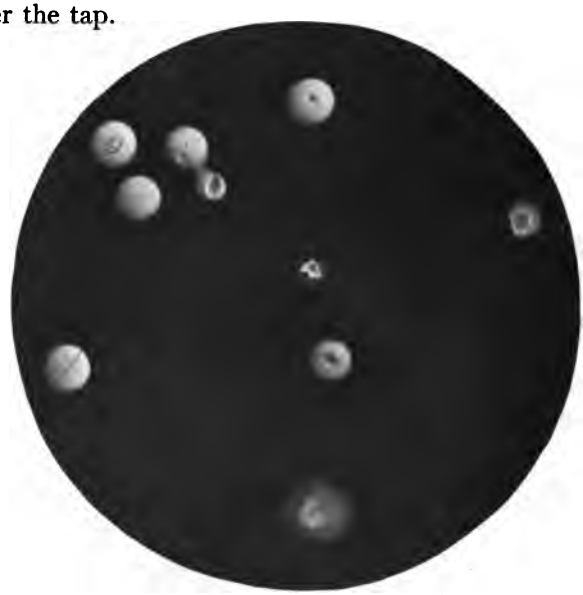


Fig. 4.—Plate colonies of Bacillus radicicola from clover, after ten days at 28° C.; × 2.

2. Compare the number, size, and position of the nodules on the roots of these different legumes.

3. Select a large and firm nodule, cut off, and immerse for three to five minutes in mercuric chlorid solution (1 : 500), and finally in alcohol.

4. Remove alcohol by flaming and place the nodule on a sterile surface (flamed slide).

Fig. 5.—Bacillus radicicola from a root nodule of alfalfa; × 1200.

5. Cut open with a sterile knife and take out some of the inner contents.

6. Inoculate this bacterial mass from the nodule into a few drops of water in a Petri dish.

7. Make two or more loop transfers from the first Petri

dish to a second containing a few drops of water. Repeat these dilutions to a third and fourth Petri dish.

8. Pour mannit agar (m. 42) into each dish, agitate until the organisms are equally distributed, and incubate at 28° C.

9. After six to eight days examine plates. The legume colonies should be characterized by a raised moist surface and round entire form, at first glistening, later changing to an opaque white. In size these colonies vary from $1\frac{1}{2}$ to 4 mm. in diameter.

Exercise 35

Formation of Nodules by Bacillus Radicicola

1. Wash thoroughly the seeds of several legumes (alfalfa, crimson clover, red clover, etc.) and immerse in mercuric chlorid ($HgCl_2$) solution (1 : 500) for three to five minutes. For careful work, treat the seeds with mercuric chlorid in a partial vacuum.

2. Remove from mercuric chlorid solution and rinse in sterile water.

3. Next drop one or two seeds of each legume into a large tube containing soft mannit agar or a loose mass of filter-paper pulp.

4. Arrange as follows:

(*a*) 1 and 2, alfalfa uninoculated.
(*b*) 3 and 4, alfalfa inoculated with culture isolated by student.
(*c*) 5 and 6, alfalfa inoculated with culture from instructor.

In the case of large legumes—*e.g.*, soy beans—it is necessary to use vessels larger than ordinary test-tubes.

5. To inoculate, use a forty-eight-hour-old culture of the legume bacteria. Prepare a water suspension and from this take 1 c.c. for each tube.

6. Under favorable conditions nodules will begin to form in ten to fifteen days.

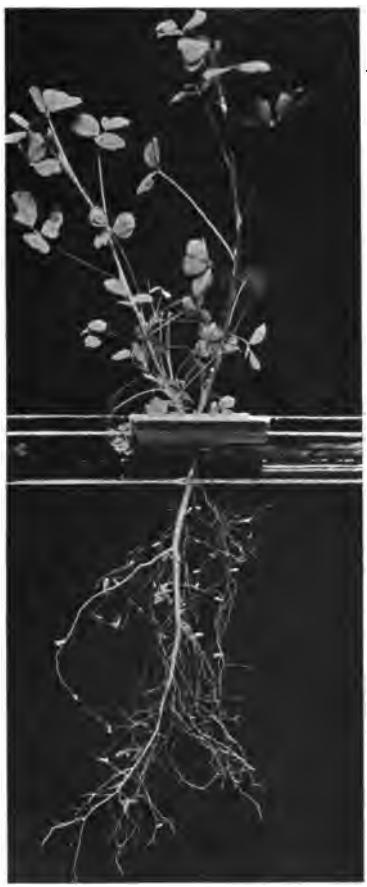


Fig. 6.—Showing inoculated alfalfa plant.

7. Keep the cultures in a warm place—greenhouse or near a window.

8. After four and six weeks examine carefully for nodules, noting the number, size, shape, and location.

Fig. 7.—Alfalfa in sterilized sand to which plant food minus nitrogen has been added.

Exercise 36

Effect of Bacillus Radicicola on the Growth and the Nitrogen Content of Alfalfa (Optional)

1. Prepare four $\frac{1}{2}$-gallon jars of clean sand and four of coal-ashes.

2. Plant to alfalfa as follows:

(*a*) 1 and 2, sand uninoculated.
(*b*) 3 and 4, sand inoculated.
(*c*) 5 and 6, coal-ashes uninoculated.
(*d*) 7 and 8, coal-ashes inoculated.

3. One week after seedlings begin to germinate add 100 c.c. per jar of plant food minus nitrogen.

Note.—Plant food: Add 10 grams of $CaSO_4 + 2H_2O$, 4.6 grams of KH_2PO_4, and 2.3 grams of $MgSO_4 + 7H_2O$ to 1000 c.c. of water.

4. This nutrient solution should be added at intervals of every two weeks or whenever needed. A few drops of iron chlorid or iron phosphate will be found beneficial.

5. After four to six weeks examine for nodules.

6. When mature, cut and analyze the tissue for total nitrogen.

Exercise 37

Effect of Caffein on the Formation of Bacteroids (Optional)

1. Inoculate in duplicate tubes of agar with and without caffein (m. 46).

2. Prepare caffein agar slant cultures of the following organisms: *Bacillus radicicola*, from alfalfa, pea, and vetch.

3. At regular two-day intervals study the morphology of the organisms from the different cultures.

Exercise 38

Nitrogen Fixation by Bacillus Radicicola in Solution

1. Prepare four 500-c.c. Erlenmeyer flasks with 100 c.c. each of mannit soil extract (m. 43).

2. Arrange as follows:

(*a*) 1 and 2, uninoculated.
(*b*) 3 and 4, inoculated with a pure culture of alfalfa bacteria.

3. Incubate at 28° C. for three weeks.
4. Then analyze for total nitrogen.

Exercise 39

Production of Gum by Bacillus Radicicola

1. Inoculate two tubes of different media (42 and 47) with *Bacillus radicicola*, and at the same time leave two tubes uninoculated.
2. Incubate for four to five weeks at 28° C., and test for gum.
3. Add 10 c.c. of alcohol (95 per cent.) or 5 c.c. of acetone to each tube.
4. Note the precipitation of gum.

Exercise 40

Artificial Cultures for the Inoculation of Legumes

A. 1. Inoculate one bottle of mannit agar (m. 42) and one of mannit solution with a pure culture of legume bacteria. Use 500-c.c. bottles with flat sides. In the liquid culture take 300 c.c. of the medium; in the agar culture, 100 c.c. Dilute a young legume culture of the desired organism with 5 c.c. of sterile water. Shake thoroughly and pipet 1-c.c. portions into the bottles of liquid and solid media. Be sure that the inoculum covers the entire surface of the agar.
2. Incubate the cultures at 28° C. for four to ten days.
3. *Determine the number of bacteria in each bottle.* The liquid culture may be treated as follows: Shake thoroughly, remove 1 c.c. to a 99-c.c. water blank, and continue the dilution to 1 : 1,000,000 and 1 : 10,000,000. Pour mannit agar plates.

Follow the same procedure with the solid culture, adding 200 c.c. of sterile water to the agar. Shake thoroughly, dilute, and plate as given in the preceding directions.

B. 1. Place about ten bacteria-free seeds in each culture bottle. Shake and pour off the liquid.

2. With sterile forceps remove about five seeds to a sterile Petri dish.

3. Allow seeds to dry in the Petri dish.

4. After twenty-four to forty-eight hours count the number of legume bacteria on each seed.

5. Place the seeds in a 100-c.c. water blank, shake, and plate 1-c.c. portions.

Exercise 41

Nitrogen Content of Bacteria (Optional)

1. Grow a mass culture of bacteria in a large Petri dish or pan. Any vigorous growing organism may be used, *e. g.*, Azotobacter.

Note.—Use 2 to 2.5 per cent. agar.

2. When cool, inoculate the surface of the agar with 5 c.c. of a rich suspension of Azotobacter in sterile water.

3. Incubate ten to fifteen days.

4. At the end of this time remove growth by carefully scraping off with a clean glass slide.

5. Dry the mass culture at 100° C. and pulverize by grinding in a mortar.

6. Analyze for total nitrogen. If desirable, a portion of the material may be saved for further analysis—*e. g.*, potassium and phosphorus.

SECTION III

RELATION OF MICROÖRGANISMS TO THE CARBON CYCLE

Exercise 1

Fermentation of Cellulose in Impure Cultures (Liquid)

1. FILL six large test-tubes about half-full of Omelianski's solution (m. 48).
2. Add four strips of filter-paper to each tube.
3. Treat as follows:

   (*a*) 1 and 2, uninoculated.
   (*b*) 3 and 4, stable manure.
   (*c*) 5 and 6, garden soil.

4. Incubate at 28° C.
5. Cover the solution in tubes 1, 3, and 5 with $\frac{1}{2}$-inch layer of paraffin oil.
6. Examine the cultures at regular intervals, taking note of the changes in the filter-paper.
7. When the filter-paper shows the first evidences of disintegration, make transfers to new tubes of Omelianski's medium.

Exercise 2

Fermentation of Cellulose in Impure Cultures (Soil)

1. Place 200-gram portions of garden soil in three 1-liter Erlenmeyer flasks.

2. Treat as follows:

(*a*) Control.
(*b*) Add 1 per cent. of sugar.
(*c*) Add 1 per cent. of green clover.

3. Bring the moisture content of the soil up to two-thirds saturation.

4. Insert one round filter-paper, diameter smaller than that of the flask, into each culture. Partially cover the filter-paper with soil.

5. Incubate at 28° C.

6. At weekly intervals examine. Note the change in the filter-paper.

Exercise 3

Fermentation of Cellulose in Soil

1. Prepare four large soup plates with 500 grams each of soil. Use two plates of field and two plates of garden soil.

2. Add to each plate 5 grams of dry filter-paper cut into strips about 2 inches long and $\frac{1}{4}$ inch wide. These should be thoroughly mixed with the soil and a little more water than necessary for half-saturation added.

3. In order to avoid rapid evaporation cover with inverted plates and keep the moisture constant.

4. After six to eight weeks remove the remaining paper from the soil by passing the soil through a fine mesh sieve. Now wash the paper, dry, and weigh. Although this procedure does not always give uniform results, it will show the relative cellulose-destroying power of the soil.

Exercise 4

Fermentation of Cellulose by Denitrifying Bacteria

1. Fill completely two Erlenmeyer flasks of 200-c.c. capacity each with medium 37.

2. Inoculate as follows:

(*a*) 1 c.c. of a rich sewage suspension.
(*b*) 1 gram of garden soil.

3. Incubate at 35° C. in a pan or plate so arranged as to catch the overflow from the flasks. After one week the cultures should begin to show fermentation, and by the end of the second or third week all nitrates should be destroyed.

4. When the solution no longer reacts for nitrates pour the turbid liquid off without removing the paper. Refill the flask with the same medium minus the paper. Now the process should proceed very much faster than in the case of the first inoculation.

5. The addition of new culture-media may be repeated several times.

Exercise 5

Isolation of Cellulose Bacteria

A. *Without Enrichment Cultures:*

1. Dilute samples of soil in such a way that plates may be made representing various dilutions, about 1 : 10,000 and 1 : 100,000 of a gram of the original material.

2. Garden, field, and marsh soil should be used.

3. Plate all of these inocula with medium 52.

B. *With Enrichment Cultures:*

1. At the same time plates are poured for A make transfers from the enrichment culture, Exercise 1, in sterile 99-c.c. water blanks.

2. From the first 99-c.c. water blank make 1-c.c. transfer to a second and a third.

3. Pour cellulose agar plates from the second and third dilution.

4. Incubate all of the plates under a bell jar at 28° C. Very often the cellulose organisms do not appear for several weeks.

5. Look for the colonies with clear zones.

Exercise 6

Formation of Carbon Dioxid from Organic Substances

1. For this exercise use a field soil and adjust the moisture content to about half-saturation.

2. Mix thoroughly so as to have the entire sample uniform.

3. Weigh into suction flasks (2-liter capacity) four equal quantities of the soil, 1 kilo each, and treat as follows:

(*a*) Untreated.
(*b*) Add 2 per cent. of finely ground vegetable matter, clover, corn, beet leaves, or something similar.
(*c*) Add 2 per cent. of air-dried and finely ground barnyard manure.
(*d*) Add 1 per cent. of cane-sugar.

4. Connect to a glass cylinder so arranged with glass beads and alkali that the carbon dioxid (CO_2) will be caught as it is drawn through the solution.

5. Every forty-eight hours determine the amount of carbon dioxid by drawing a current of air through the apparatus for twenty minutes with a water-pump (see page 150). Free the current of air from carbon dioxid by passing through strong N/1 potassium hydroxid. In order to regulate the air, count the number of air bubbles.

6. Allow the experiment to run for twelve days.
7. Arrange the results in a table.

TABLE 28.—*The Influence of Organic Substances on the Evolution of Carbon Dioxid from Soil*

Two-day periods.	Carbon dioxid in 100 gm. of soil.			
	Control.	Clover 2 per cent.	Barnyard manure 2 per cent.	Cane-sugar 2 per cent.
	Mgm.	Mgm.	Mgm.	Mgm.
1				
2				
3				
4				
5				
6				
Total				

Exercise 7

Formation of Humus in Soil (Optional)

1. Prepare a uniform sample of soil (air-dry) by passing through a sieve.
2. Weigh out 500-gram portions and treat as follows:

(*a*) Control.
(*b*) Add 2 per cent. finely ground wheat straw.
(*c*) Add 2 per cent. finely ground alfalfa.
(*d*) Add 2 per cent. finely ground dry manure.
(*e*) Sterile, no additional treatment.
(*f*) Add 2 per cent. finely ground wheat straw; sterilize.
(*g*) Add 2 per cent. finely ground alfalfa; sterilize.
(*h*) Add 2 per cent. finely ground dry manure; sterilize.

3. Pint or quart Mason jars can be used as receptacles for the various soils.

4. Add sufficient sterile water to bring moisture content up to two-thirds saturation.

5. Cover loosely with a Petri dish and incubate three to four months, restoring moisture from time to time.

6. At the end of this time prepare the cultures for analysis.

7. Dry and determine the amount of humus in 10-gram samples (see page 148).

SECTION IV

RELATION OF MICROÖRGANISMS TO THE SULPHUR CYCLE

Exercise 1

Reduction of Sulphates with the Formation of Hydrogen Sulphid

1. PREPARE three small bottles of sulphate solution (m. 55).

2. Inoculate as follows:

(*a*) Uninoculated.
(*b*) 1 gram of rich soil.
(*c*) 1 c.c. of sewage slime.

3. Stopper tightly with paraffined corks.

4. Incubate at 28° C. for two or four weeks.

5. At the end of this time remove bottles from incubator; note the change in color and odor.

6. Hold over the open mouth of the bottle a small piece of filter-paper saturated with a solution of lead acetate. A blackening of the paper shows the presence of hydrogen sulphid.

7. Remove a few cubic centimeters with a pipet to a test-tube or small Erlenmeyer flask.

8. Add a few drops of $BaCl_2$ solution.

9. Compare the amount of white precipitate in the inoculated cultures with that in the uninoculated control.

10. The amount of hydrogen sulphid may be determined quantitatively by titrating with iodin and sodium thiosulphate (see page 154).

Exercise 2

Isolation of Hydrogen Sulphid Organisms

1. Prepare four tubes of sulphate-reducing gelatin (m. 56).

2. Inoculate shake cultures of the gelatin with various dilutions of the impure cultures from the previous exercise.

3. Harden the gelatin cultures in cold water and incubate at room temperature four to seven days in an anaërobic jar (see page 133).

4. In case black colonies appear in the tubes, attempt to isolate the organisms by making subinoculations into the sulphate gelatin.

Exercise 3

Hydrogen Sulphid from Protein and Sulphur (Optional)

1. Prepare two small bottles of culture solutions (1) and (2) (m. 59).

2. Treat as follows:

(*a*) Solution 1, uninoculated.
(*b*) Solution 1, 1 gram garden soil.
(*c*) Solution 2, uninoculated.
(*d*) Solution 2, 1 gram garden soil.

3. Compare the changes that take place in solutions (1) and (2).

Exercise 4

Oxidation of Thiosulphates

1. Place in four flasks (300-c.c. Erlenmeyer) about 20 c.c. each of Nathansohn's solution (m. 60).

2. Inoculate two flasks with 0.1 gram of soil each. Leave two flasks uninoculated.

3. Incubate for four weeks at 28° C.

4. Remove the flasks from incubator and test for sulphates.

SECTION V

RELATION OF MICROÖRGANISMS TO THE IRON-CYCLE

Exercise 1

A Method for Growing Crenothrix and Spirophyllum

1. CLEAN and sterilize a Berkefeld filter.

2. Connect the filter to the city water-supply and allow the water to run slowly for twenty-four hours.

3. Remove the metal cap from the filter and place in a large beaker of iron solution (m. 61).

4. Incubate in the ice-box or at 15° to 20° C.

5. At regular two-day intervals examine the deposit on the sides of the filter.

6. If bacteria are found, test for iron. Add a few drops of a 5 per cent. hydrochloric acid solution and a 4 per cent. potassium ferrocyanid solution. In the presence of ferric salts an intense blue color is formed.

7. In order to stain the higher forms of iron bacteria it is well to remove the deposit of iron by treating with a 5 per cent. hydrochloric acid solution.

Exercise 2

Iron Precipitating Bacteria

1. Shake 20 grams of field soil with 200 c.c. of water. Dilute until 1 c.c. equals 1 : 100,000.

2. Pour plates with medium 64.

3. Incubate the plates for several weeks at 28° C.

4. Note the precipitation of iron compounds around certain colonies.

A

B

Fig. 8.—Iron bacteria: *A*, Crenothrix thread showing germination of spores within sheath; × 850. *B*, Chlamydothrix showing simple and curved threads; × 850.

A

B

Fig. 9.—Iron bacteria: *A*, Gallionella, Chlamydothrix, and Spirophyllum; × 850. *B*, Spirophyllum; × 850.

SECTION VI

RELATION OF MICROÖRGANISMS TO THE PHYSICAL PROPERTIES OF SOIL

Exercise 1

Movements of Soil Water

1. PREPARE four glass cylinders or large test-tubes as follows: Fill two three-fourths full of quartz sand and two three-fourths full of soil.

2. Treat as follows:

(*a*) Sand plus 1 per cent. of sugar bouillon previously inoculated with 5 c.c. of a rich soil suspension.
(*b*) Sand plus 1 per cent. of sugar bouillon previously inoculated with 5 c.c. of a rich soil suspension.
(*c*) Soil plus 1 per cent. sugar.
(*d*) Soil plus 1 per cent. sugar.

3. Add enough bouillon to the sand and enough water to the soil to completely saturate the columns.

4. In order to prevent bacterial growth add 5 c.c. of a 1 : 5 mercuric chlorid solution to cylinders (*a*) and (*c*), and the same amount of water to cylinders (*b*) and (*d*).

5. Mark on the cylinders the height of the column of water.

6. Incubate at 28° C., and examine each day.

FORMULÆ AND METHODS

CLEANING GLASSWARE

1. ALL glassware must be thoroughly cleaned before it is ready to use. Test-tubes, Petri dishes, flasks, and similar glassware should be boiled in a 5 per cent. soda solution or washed in hot soapsuds until free from organic matter. When it is desirable to use very clean glassware, immerse for ten minutes or longer if possible in the dichromate solution.

Potassium ($K_2Cr_2O_7$) or sodium dichromate ($Na_2Cr_2O_7$)...	80 gm.
Water.....	300 c.c.
Sulphuric acid (H_2SO_4).....	460 c.c.

Note. - Dissolve the dichromate in warm water and, when cool, add slowly concentrated sulphuric acid. If properly prepared, the liquid should be thick, with small crystals. It may be used repeatedly, provided the crystals are present.

2. After removing from the cleaning solution rinse thoroughly in distilled water.

3. Dirty cover-glasses and slides may be treated in the same manner. Drop these, one at a time, into the dichromate mixture and allow to remain for several hours. Remove from this solution, wash, and wipe with a soft, clean cloth.

4. A simple and more rapid method, suitable for general work, is to rub the slides with moist Bon Ami, and when dry to polish them with a clean cloth.

5. In order to remove fat pass the cover-slips through a flame. Where it is desirable to have very clean slides and

cover-slips it is well to heat them in water and then in 50 per cent. sulphuric acid. After rinsing in distilled water, wash in alcohol and wipe with a clean cloth. These should be kept in a clean, covered dish.

SECTION VII

PREPARATION OF CULTURE-MEDIA

The culture-media are arranged according to the groups of soil microörganisms. Since it is not possible to grow all of the strains of bacteria in one group on the same medium, several formulæ are given.

MEDIA FOR THE DETERMINATION OF THE NUMBER AND FOR THE SEPARATION OF SOIL BACTERIA

Medium 1

Bouillon or Nutrient Broth

Peptone	10 gm.
Liebig's meat extract	3 gm.
Distilled water	1000 c.c.

1. Add to 1 liter of distilled water 3 grams of meat extract and 10 grams of peptone.
2. Record the weight of vessel and contents.
3. Heat *not above* 50° C. in steamer or double boiler until extract and peptone are dissolved.
4. Titrate and adjust reaction to 1 per cent. acid, with phenolphthalein as an indicator (+1).
5. Boil over the free flame for fifteen minutes.
6. Restore the loss in weight with distilled water.
7. Titrate again.

8. Sterilize broth in large flask and allow to stand until next laboratory period.

9. Refilter through fine paper and tube.

10. Fill tubes about one-third full.

11. Plug the tubes and sterilize in autoclave at 120° C. for fifteen minutes.

Note.—To titrate, remove 5 c.c. of the medium to a casserole or small flask containing about 45 c.c. of distilled water. Boil one minute with constant stirring, add 3 drops phenolphthalein, and neutralize excess acid with N/20 NaOH. If 1 c.c. N/20 NaOH is required to neutralize 5 c.c. of the medium, the reaction is correct. In this way calculate the amount of normal alkali or acid necessary to adjust the reaction of the entire bulk of culture-medium to 1 per cent. (+1).

All reactions must be expressed with reference to the phenolphthalein neutral point. They are stated in percentages of normal acid or alkaline solutions required to neutralize them (Fuller's scale). Alkaline media should be recorded with the minus sign (—) before the percentage of normal acid needed for their neutralization; acid media should be written with the plus sign (+) before the percentage of normal alkaline solution necessary for their neutralization.

The example below will illustrate the method. If the required reaction is (+1) and the buret reading shows that 1.8 c.c. of N/20 NaOH has been used in neutralizing the 5 c.c. of broth, then the problem may be stated as follows:

5 c.c. of broth require the addition of 1.8 c.c. N/20 NaOH to neutralize it.

100 c.c. of broth require the addition of 36 c.c. N/20 NaOH or 1.8 c.c. N/1 NaOH to neutralize it.

1000 c.c. of broth require the addition of 18 c.c. N/1 NaOH to neutralize it.

The figures above show that the broth as titrated is 0.8 per cent. too acid, and that 8 c.c. of normal NaOH per liter must be added to obtain the proper reaction.

Do not neutralize medium first and then readjust by the addition of acid. This tends to precipitate certain substances which are favorable to bacterial development.

The broth prepared in this way should be of a golden color and should not develop a precipitate upon subsequent sterilization in the autoclave. Adjust reaction by adding normal hydrochloric acid or sodium hydroxid.

The hydrogen electrode may be used to determine the reaction of culture-media.

Fuller, G. W., Jour. Pub. Health Assoc., vol. xx, pp. 381–399, 1895.
Standard Methods of Water Analysis, 1915.
Clark, W. M., Jour. Inf. Diseases, vol. xvii, pp. 109–136, 1915.
Anthony and Ekroth, Jour. Bact., vol. i, pp. 230–232, 1916.
Itano, A., Bul. 167, Mass. Agr. Exp. Sta., 1916.

12. *Titration of Broth.*—Titrate the two samples of broth prepared by the instructor. Determine the amount of N/1 alkali or acid required to make 1 liter of these solutions (+1). Do this for each sample. Titrate sample No. 2, hot and cold, using phenolphthalein and litmus as indicators. Record results.

Medium 2

Nutrient Gelatin

Gelatin	100 to 150 gm.
Liebig's meat extract	3 gm.
Peptone	10 gm.
Distilled water	1000 c.c.

1. In a convenient vessel measure 1000 c.c. of nutrient broth.

2. Add 10 per cent., on the *dry* basis, of gold label sheet gelatin. Let the gelatin soak five to ten minutes.

3. Heat over water-bath until dissolved.

4. Adjust the reaction as directed in the preparation of nutrient broth. Gelatin is decidedly acid and will require more NaOH to neutralize it than bouillon or agar.

5. Cool this mass to about 60° C. Add the whites of two eggs or 3 grams of powdered egg-albumen to 25 c.c. of water. Stir into the gelatin and heat in a double boiler. The egg-albumen will coagulate and inclose most of the

impurities. When this coagulum has settled to the bottom, pour the cleared gelatin through the filter.

6. If properly prepared, gelatin may be filtered through filter-paper. Otherwise it will be necessary to use an absorbent cotton filter.

Note.—A cotton filter is prepared as follows: In the base of a large funnel place a small amount of clean excelsior. In place of the excelsior a small spiral of copper wire may be used. On top of this put two or three layers of absorbent cotton. Split a piece of absorbent cotton, somewhat larger than the top of the funnel, horizontally into two layers of equal thickness. Place one layer of cotton above the other, so that the fibers are at right angles. Pour the medium, slowly at first, on to the filter. (In order to avoid breaking the filter use a glass rod to direct the fluid to the center of the filter.) When the filtrate begins to come through the cotton, fill the funnel. If the first filtrate is not clear, the turbid liquid should be refiltered through the same cotton.

7. Sterilize in the autoclave for ten minutes at 120° C.

8. As soon as removed from the autoclave stand in cold or ice-water. Gelatin is easily decomposed, and if heated too high or too long will not solidify.

Medium 3

Nutrient Agar

Agar	15 gm.
Liebig's meat extract	3 gm.
Peptone	10 gm.
Distilled water	1000 c.c.

1. In a vessel containing 1000 c.c. of water add 15 grams of thread agar.

2. Heat in the steamer or double boiler until the agar is dissolved. This requires at least one hour.

3. Add 3 grams extract of meat and 10 grams of peptone.

4. When completely dissolved adjust the reaction to (+1).

5. Clear with egg-albumen and filter as directed under the preparation of gelatin.

6. Tube, and sterilize in the autoclave for fifteen minutes. The amount of agar to place in tubes will depend on the purpose for which the agar is to be used. For slants, about 5 c.c. is enough; for plates, about 10 c.c.

Note.—For making especially clear agar, adjust the reaction to (+1.5) with N/1 HCl before adding the agg-albumen. Heat in the steamer until the albumen is coagulated and settled to the bottom of the dish. If it will not settle, stir the agar vigorously and continue heating. It may be three or four hours before it is ready to be filtered. Filtering then consists only in decanting the cleared agar through either a cotton filter or filter-paper. Do not pour the dirt and albumen on to the filter. Titrate and adjust the reaction to (+1).

Medium 4

Heyden-Nährstoff Agar

Agar	12.0 gm.
Heyden-Nährstoff	7.5 gm.
Distilled water	1000.0 c.c.

1. To 500 c.c. of cold distilled water in a flask add 7.5 grams of Heyden-Nährstoff. Shake until a good suspension is obtained and allow the mixture to stand for thirty minutes or more.

2. Heat in steamer or double boiler for one hour, or until the upper portion of the solution is clear.

3. While hot filter through paper.

4. Dissolve 12 grams of agar in 500 c.c. of water. Filter and mix the Heyden-Nährstoff and agar solutions.

5. *It is not necessary to adjust the reaction of this medium.*

Heyden-Nährstoff, The Heyden Chemical Works, 135 Williams St., New York City, N. Y.

Medium 5

Casein Agar

Agar	10 gm.
Casein	10 gm.
Sodium hydroxid N/1 (NaOH)	7 c.c.
Distilled water	1000 c.c.

1. Measure into an Erlenmeyer flask 100 c.c. of distilled water and 10 grams of casein (Hammarsten).

2. Add to this 7 c.c. of normal NaOH.

3. Heat in a double boiler or steamer to get a perfect solution.

4. Dissolve 10 grams of agar in 900 c.c. of distilled water.

5. Mix and filter the casein-agar solution.

6. Adjust the reaction between (+0.1) and (+0.2) per cent. If the casein is weighed accurately and the normal solution is correct, the reaction will be about (+0.2).

7. Tube, and sterilize in autoclave for fifteen minutes. Cool quickly in cold or iced water.

Note.—The final reaction of the medium will be about (+0.1), Fuller's scale. If the medium is alkaline, the bacterial growth will be restricted. If the medium is more than (+0.1) some of the casein may be precipitated during sterilization. The casein agar should be clear and almost colorless when poured into a Petri dish. Sometimes the casein will be slightly precipitated during the sterilization or the cooling. This is of no consequence, since the precipitate, when poured into plates, is so finely divided that it becomes invisible. Casein agar should be incubated for six days at 30° C.

Ayres, S. H., United States Dept. Agr. Bur. Animal Indus., 28th Ann. Report, pp. 225–235, 1911.

Medium 6

Soil-extract Agar

Agar	15 gm.
Dextrose ($C_6H_{12}O_6$)	1 gm.
Soil extract	100 c.c.
Water	900 c.c.

1. Dissolve the agar in 900 c.c. of water by heating in the steamer for one hour or longer. Add 100 c.c. of the stock soil-extract solution.

2. Add the dextrose just prior to tubing.

3. The reaction should be (+0.5) or nearly neutral.

Note.—Stock Solution of Soil Extract.—This is prepared by heating 1000 grams of garden soil with 1000 c.c. of tap-water in the autoclave at 5 to 10 pounds' pressure for thirty minutes. A small amount of calcium carbonate is added and the whole is filtered through a double paper filter. The turbid filtrate should be poured back on to the filter until it comes through clear.

Medium 7

Soil-extract Gelatin

Gelatin	100 to 150 gm.
Dextrose ($C_6H_{12}O_6$)	1 gm.
Soil extract	100 c.c.
Water	900 c.c.

1. Dissolve the gelatin in the diluted soil-extract solution by heating slowly in the steamer.

2. Clarify the medium with egg-albumen.

3. Add 1 gram of dextrose and adjust the reaction to (+0.5).

Conn, H. J., Bul. 38, N. Y. Agr. Exp. Sta., 1914.

Medium 8

Asparagin-dextrose Agar for Soil Bacteria

Agar	15.0 gm.
Dibasic potassium phosphate (K_2HPO_4)	0.5 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	0.2 gm.
Asparagin ($C_4H_8N_2O_3 + H_2O$)	1.0 gm.
Dextrose ($C_6H_{12}O_6$)	1.0 gm.
Distilled water	1000.0 c.c.

1. After dissolving the agar by steaming for one hour or more, add the dibasic potassium phosphate and magnesium sulphate.

2. The asparagin and dextrose should be added just before sterilizing.

3. Adjust the reaction to (+1.0).

Medium 9

Urea-ammonium Nitrate Agar for Soil Bacteria

Agar	15.0 gm.
Dibasic potassium phosphate (K_2HPO_4)	0.5 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	0.2 gm.
Dextrose ($C_6H_{12}O_6$)	10.0 gm.
Urea ($CO(NH_2)_2$)	0.05 gm.
Ammonium nitrate (NH_4NO_3)	0.1 gm.
Ferric sulphate ($Fe_2(SO_4)_3$)	trace
Distilled water	1000.0 c.c.

The urea, ammonium nitrate, and dextrose should not be added until the medium is ready for sterilization. The reaction should be about (+0.25).

Cook, R. C., Soil Science, vol. i, No. 2, pp. 153–161, 1916.

Medium 10

Sodium Asparaginate Agar for Soil Bacteria

Agar	12.0 gm.
Ammonium biphosphate ($(NH_4)H_2PO_4$)	1.5 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	0.2 gm.
Sodium asparaginate ($NaC_4H_6NO_4 + H_2O$)	1.0 gm.
Dextrose ($C_6H_{12}O_6$)	1.0 gm.
Calcium chlorid ($CaCl_2$)	0.1 gm.
Potassium chlorid (KCl)	0.1 gm.
Ferric chlorid ($FeCl_3 + 6H_2O$)	trace
Distilled water	1000.0 c.c.

The sodium asparaginate and dextrose should not be added until the medium is ready for sterilization. The reaction

should be between (+0.8 or +1.0). It requires about 10 c.c. of N/1 NaOH per liter. In place of clarifying with the white of egg, the author recommends heating for half an hour at 15 pounds' pressure without disturbing the sediment and decanting through a cotton filter.

Conn, H. J., Bul. 38, New York Agr. Exp. Sta., 1914.

COUNTING SOIL PROTOZOA

Medium 11

Hay-soil Extract

Soil extract	100 c.c.
Hay extract (0.1 per cent. of dry hay)	100 c.c.
Calcium carbonate ($CaCO_3$)	5 gm.
Tap-water	800 c.c.

Medium 12

Hay Infusion

Hay	10 gm.
Tap-water	1000 c.c.

Medium 13

Hay Egg-albumen (Ciliates)

Hay	100 gm.
Egg-albumen	50 gm.
Tap-water	1000 c.c.

Medium 14

Soil Extract (Flagellates and Ciliates)

Soil extract	100.0 c.c.
Dibasic potassium phosphate (K_2HPO_4)	0.5 gm.
Tap-water	900.0 c.c.

Medium 15

Mannit Solution

See Culture-medium No. 39, page 108.

AMMONIFICATION

Medium 16

Peptone Solution

Peptone	10 gm.
Distilled water	1000 c.c.

Heat in the autoclave for thirty minutes and filter.

Medium 17

Gelatin Solution

Gelatin	5 gm.
Distilled water	1000 c.c.

Bring the reaction to (+1.0).

Medium 18

Casein Solution

Casein	10 gm.
Normal sodium hydroxid (NaOH)	7 c.c.
Distilled water	1000 c.c.

Prepare according to directions on page 94.

Medium 19

Urea Solution

Urea ($CO(NH_2)_2$)	20 gm.
Bouillon (—1.0)	1000 c.c.

1. Heat in the steamer or over a free flame until the precipitate settles.

2. Filter and sterilize.

3. In order to diminish the loss of ammonia from urea the culture-media should be sterilized in the steamer twenty minutes on three successive days.

Medium 20

Urea Solution

Certain forms of urea fermenters prefer a medium richer in urea. This may be prepared by adding 10 per cent. of urea to the bouillon.

Medium 21

Urea Solution

Urea ($CO(NH_2)_2$)	30.0 gm.
Dibasic potassium phosphate (K_2HPO_4)	0.5 gm.
Calcium citrate ($Ca_3(C_6H_5O_7)_2 + 4H_2O$)	10.0 gm.
Tap-water	1000.0 c.c.

Söhngen, N. L., Centbl. Bakt. (etc.), Abt. 2, Bd. 23, p. 94, 1909.

Medium 22

Urea Solution

Urea ($CO(NH_2)_2$)	50.0 gm.
Dibasic potassium phosphate (K_2HPO_4)	0.5 gm.
Soil extract	100.0 c.c.
Tap-water	900.0 c.c.

Löhnis, F., Centbl. Bakt. (etc.), Abt. 2, Bd. 14, p. 714, 1905.

Medium 23

Urea Gelatin

Gelatin	120 to 150 gm.
Urea ($CO(NH_2)_2$)	20 c.c.
Bouillon (—1.0)	1000 c.c.

The urea decreases the solidifying properties of gelatin. If agar medium is wanted, take 15 grams to 1 liter.

Medium 24

Hippuric Acid Solution

Sodium hippurate ($NaC_9H_8NO_3$)	3.0 gm.
Dibasic potassium phosphate (K_2HPO_4)	0.5 gm.
Tap-water	1000.0 c.c.

Medium 25

Uric Acid Solution

Uric acid ($C_5H_4N_4O_3$)	3.0 gm.
Dibasic potassium phosphate (K_2HPO_4)	0.5 gm.
Tap-water	1000.0 c.c.

Löhnis, F., Landwirtschaftliche-bakteriologisches Praktikum, Berlin, pp. 112, 113, 1911.

NITRIFICATION

Medium 26

Solution for Nitrite Formation

Ammonium sulphate ($(NH_4)_2SO_4$)	1.0 gm.
Dibasic potassium phosphate (K_2HPO_4)	1.0 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	0.5 gm.
Sodium chlorid (NaCl)	2.0 gm.
Ferrous sulphate ($FeSO_4 + 7H_2O$)	0.4 gm.
Magnesium carbonate ($MgCO_3$) in excess	about 5.0 gm.
Distilled water	1000.0 c.c.

In order to prevent any loss of ammonia, it is well to sterilize the ammonium sulphate separately. A 10 per cent. solution will be found very convenient. When cool, the proper amount of ammonium sulphate may be added with a sterile pipet.

Winogradsky, Lafar, Technische Mykologie, Bd. 3, pp. 132–181, 1904.

Medium 27

Solution for Nitrite Formation

Magnesium ammonium phosphate ($Mg(NH_4)PO_4 + 6H_2O$)	2.0 gm.
Dibasic potassium phosphate (K_2HPO_4)	0.5 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	0.5 gm.
Sodium chlorid (NaCl)	1.0 gm.
Ferrous sulphate ($FeSO_4 + 7H_2O$)	0.4 gm.
Magnesium carbonate ($MgCO_3$)	5.0 gm.
Distilled water	1000.0 c.c.

It is not necessary to sterilize the magnesium ammonium phosphate separately.

Medium 28

Solution for Nitrate Formation

Sodium nitrite ($NaNO_2$)	1.0 gm.
Dibasic potassium phosphate (K_2HPO_4)	0.5 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	0.3 gm.
Sodium chlorid (NaCl)	0.5 gm.
Ferrous sulphate ($FeSO_4 + 7H_2O$)	0.4 gm.
Sodium carbonate (Na_2CO_3) (anhydrous)	0.3 gm.
Distilled water	1000.0 c.c.

The formation of nitrates takes place rapidly, provided the cultures are grown under conditions that supply an abundance of oxygen.

Medium 29

Solution for Nitrite and Nitrate Formation

Ammonium sulphate ($(NH_4)_2SO_4$)	2.0 gm.
Dibasic potassium phosphate (K_2HPO_4)	1.0 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	0.5 gm.
Sodium chlorid (NaCl)	2.0 gm.
Ferrous sulphate ($FeSO_4 + 7H_2O$)	0.4 gm.
Calcium carbonate ($CaCO_3$)	5.0 gm.
Distilled water	1000.0 c.c.

The ammonium sulphate or the calcium carbonate should be sterilized separately and added after cooling. In place of 2 grams of ammonium sulphate, 7.4 grams of magnesium ammonium phosphate may be used. This solution is suited to a quantitative study of nitrification.

Medium 30

Silicate Jelly for the Nitrifying Bacteria

A. *Undialyzed*

1. Prepare a solution of sodium silicate (Na_2SiO_3) of approximately 8 per cent. Weigh out the sodium silicate and boil in water for thirty minutes, filter through cotton and coarse grained filter-paper. It is more convenient to use Merck's concentrated solution of sodium silicate and dilute to the desired strength. This must be kept tightly stoppered.

2. Prepare a solution of hydrochloric acid (HCl) of such a strength that 1 c.c. of the acid neutralizes 1 c.c. of the sodium silicate, using methyl-orange as an indicator; or use normal hydrochloric acid and determine the amount of sodium silicate required to neutralize the acid.

3. To 120 c.c. of the hydrochloric acid solution add, with stirring, 100 c.c. of the sodium silicate solution. If normal acid is used, be sure there is 20 c.c. excess of acid in each 220 c.c. of the mixture.

4. Tube 12-c.c. portions of the mixture and sterilize in the autoclave for ten minutes at 15 pounds' pressure. If the tubes are sealed tightly this mixture may be kept for several weeks. In case the mixture becomes a milky color or solid when taken from the autoclave, it indicates an

insufficient quantity of hydrochloric acid was added. Repeat, using more acid.

5. Prepare a solution containing the nutrient salts suitable for the growth of the desired organism. This solution should contain the salts in from 2.5 to 5 times the desired strength.

6. Tube and sterilize the nutrient solution. If 5 times normal strength is taken, use about 3 c.c. per tube, or 5 c.c. if 2.5 times normal strength.

7. Pour the silicic acid mixture into a sterile Petri dish. Inoculate the sterile nutrient solution and add a sufficient amount of a sodium carbonate solution to neutralize the excess silicic acid in the mixture and a few drops in excess. Now pour this into the dish with the silicic acid, rotate, and allow the plate to harden on an even surface. After a few moments this mixture should harden. The plates may be handled similar to agar plates. The concentration of silicic acid mixtures determines the strength of the nutrient solution to use.

A modification of the Stevens and Temple method, Centbl. Bakt. (etc.), Abt. 2, Bd. 21, pp. 84–87, 1908.

B. *Undialyzed*

1. Dissolve 8.40 grams of sodium silicate (Na_2SiO_3) and 24 grams of potassium silicate (K_2SiO_3) in 500 c.c. of distilled water. A mixture of sodium and potassium silicate decreases the sodium salt in the final medium.

2. Prepare dilute hydrochloric acid in such a way that it requires slightly more than 1 c.c. of the sodium potassium silicate solution to neutralize 1 c.c. of the HCl.

3. Add to the hydrochloric acid solution the nutrient salts suitable for the growth of the nitrifying bacteria.

4. With methyl-orange as an indicator, standardize the

hydrochloric acid mixture against the silicate so that 1 c.c. equals 1 c.c.

5. In a similar manner standardize a solution of sulphuric acid and phosphoric acid without the salts.

6. The three acids should then be mixed. Approximately, 1 c.c. of the acid mixture will neutralize 1 c.c. of the silicate mixture.

Doryland, C. J. T., Jour. of Bact., vol. i, No. 2, pp. 143–148, 1916.

C. *Partially Dialyzed*

1. Make a solution of sodium silicate as in the undialyzed procedure.

2. Mix with approximately normal hydrochloric acid, making the mixture decidedly acid.

3. Dialyze in running water, using parchment, animal membrane, or collodion sacs until nearly all the chlorids have disappeared. Make sure all the chlorids do not dialyze out, or the mass will solidify.

Note.—Collodion is conveniently prepared by dissolving soluble guncotton in a mixture of equal parts of 95 per cent. alcohol and sulphuric ether. Take about 5 grams of clean, white guncotton per 100 c.c. of fluid. It requires at least twenty-four hours to completely dissolve the guncotton.

Pour the collodion slowly into clean test-tubes and rotate. Try to moisten the interior of the tube without forming air bubbles. The excess of collodion should be poured back into the bottle and the tube slowly rotated in order to keep the interior of the tube covered with a uniform layer. After pouring off the excess, stand the tube upright, mouth down, on a sheet of clean paper to drain. Wipe off the excess of collodion from about the mouth of the tube. Now rotate the tube for five minutes or more with the mouth in a draft. When dry, remove the sac by cutting around mouth of tube and filling with water. Allow the collodion sacs to stand in water until ready to use.

Fill the sacs with the acid sodium silicate and tie the mouth with rubber bands. When dialyzed, pour the silicate jelly out of the collodion sacs into a clean beaker and boil for one or two minutes over an open flame. This should remove the absorbed air.

4. Make the mixture 10 per cent. acid with strong hydrochloric acid.

5. Tube and sterilize as before, and proceed as with the undialyzed media.

6. In case the silicic acid does not solidify, the dialyzed solution may be concentrated.

Medium 31

Washed Agar for the Nitrifying Bacteria

1. Heat ordinary agar with distilled water until in solution.

2. Pour into Erlenmeyer flasks and allow to solidify.

3. After standing for one or two weeks with several changes of water, all the soluble organic substances will have been removed.

4. Add the inorganic substances and sterilize. It is well to use precipitated calcium carbonate and hydrogen ammonium sodium phosphate ($NH_4NaHPO_4 + 4H_2O$).

Medium 32

Magnesium-gypsum Blocks

1. Add 1 per cent. of magnesium carbonate ($MgCO_3$) to dried gypsum ($CaSO_4 + H_2O$) and mix thoroughly.

2. To this mass add water until the mixture has the consistency of sour cream.

3. Pour upon plate glass and cut into circular blocks for Petri dishes. It is best to cut with a Petri dish of a size smaller than the dish for which it is intended. If the glass plate or Petri dish is previously washed in soapy water, the gypsum block is readily removed.

4. When hard, remove blocks from glass, place bottom

up in dishes, and add enough culture-media to half cover blocks.

5. Inoculate the surface of blocks and incubate at 25° to 30° C.

DENITRIFICATION

Medium 33

Solution for Nitrate Reduction

(*a*) Potassium nitrate (KNO_3)	1 gm.
Asparagin ($C_4H_8N_2O_3 + H_2O$)	1 gm.
Water	250 c.c.
(*b*) Citric acid ($C_6H_8O_7 + H_2O$)	5.0 gm.
or Neutral sodium citrate	8.5 gm.
Monobasic potassium phosphate (KH_2PO_4)	1.0 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	1.0 gm.
Calcium chlorid ($CaCl_2 + 6\ H_2O$)	0.2 gm.
Ferric chlorid ($FeCl_3 + 6H_2O$)	trace
Distilled water	250.0 c.c.

Neutralize the citric acid solution with a 10 per cent. solution of sodium or potassium hydroxid, using phenolphthalein as an indicator. Mix the two solutions, cool to 15° C., and add sufficient water to make 1 liter. If the asparagin and potassium nitrate are dissolved along with the other salts, a decomposition may occur. This is marked by a browning of the liquid due to the presence of nitrous acid.

For a solid medium add 15 grams of agar to 1 liter.

Medium 34

Nitrate Bouillon

Potassium nitrate (KNO_3)	5 gm.
Bouillon	1000 c.c.

Medium 35

Starch Nitrate Agar

Agar	10 gm.
Potassum nitrate (KNO_3)	1 gm.
Starch $(C_6H_{10}O_5)_n$	5 gm.
Bouillon	1000 c.c.

After colonies develop, treat one series of plates with a weak solution of potassium iodid (KI) in dilute HCl. The treatment should result in the development of a characteristic blue halo about the colonies that reduce nitrate to nitrite.

Hoffmann, C., Centbl. Bakt. (etc.), Abt. 2, Bd. 34, p. 386, 1912.

Medium 36

Solution for Nitrate Reduction

Dibasic potassium phosphate (K_2HPO_4)	0.5 gm.
Potassium nitrate (KNO_3)	10.0 gm.
Ethyl alcohol (C_2H_5OH)	5.0 c.c.
Tap-water	1000.0 c.c.

Beijerinck, M. W., Centbl. Bakt. (etc.), Abt. 2, Bd. 25, p. 35, 1910.

Medium 37

Solution for Nitrate Reduction

Dibasic potassium phosphate (K_2HPO_4)	0.5 gm.
Potassium nitrate (KNO_3)	2.5 gm.
Filter-paper in strips	20.0 gm.
Tap-water	1000.0 c.c.

Iterson, C. V., Centbl. Bakt. (etc.), Abt. 2, Bd. 11, p. 689, 1904.

Medium 38

Inorganic Solution for Nitrate Reduction

Sodium thiosulphite ($Na_2S_2O_3 + 5H_2O$)	5.0 gm.
Potassium nitrate (KNO_3)	5.0 gm.
Sodium bicarbonate ($NaHCO_3$)	1.0 gm.
Dibasic potassium phosphate (K_2HPO_4)	0.2 gm.
Magnesium chlorid ($MgCl_2 + 6H_2O$)	0.1 gm.
Calcium chlorid ($CaCl_2 + 6H_2O$)	trace
Ferric chlorid ($FeCl_3 + 6H_2O$)	trace
Distilled water	1000.0 c.c.

Lieske, R., Ber. d. deutsch. bot Gesell., Bd. 30, 1912.

NITROGEN ASSIMILATING ORGANISMS

A. *Free Nitrogen-fixing Bacteria (Aërobic)*

Medium 39

Mannit Solution

Mannit ($C_6H_8(OH)_6$)	15.0 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	0.2 gm.
Monobasic potassium phosphate (KH_2PO_4)	0.2 gm.
Sodium chlorid (NaCl)	0.2 gm.
Calcium sulphate ($CaSO_4 + 2H_2O$)	0.1 gm.
Calcium carbonate ($CaCO_3$)	5.0 gm.
Distilled water	1000.0 c.c.

Dissolve the phosphate separately in a little water and make the solution neutral to phenolphthalein with N/1 NaOH; then add to the other ingredients. For a solid medium add 15 grams of agar to each liter.

Ashby, S. F., Jour. Agr. Sci., vol. 2, p. 38, 1907.

Medium 40

Dextrose Solution

Dibasic potassium phosphate (K_2HPO_4)	0.2 gm.
Dextrose ($C_6H_{12}O_6$)	10.0 gm.
Tap-water	1000.0 c.c.

For a solid medium add 1.5 per cent. of agar.

(Anaërobic)

Medium 41

Solution for Anaërobic Organisms

Dibasic potassium phosphate (K_2HPO_4)	1.0 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	0.2 gm.
Sodium chlorid (NaCl)	0.01 gm.
Ferrous sulphate ($FeSO_4 + 7H_2O$)	0.01 gm.
Manganese sulphate ($MnSO_4 + 4H_2O$)	0.01 gm.
Dextrose ($C_6H_{12}O_6$)	20.0 gm.
Calcium carbonate ($CaCO_3$)	30.0 gm.
Distilled water	1000.0 c.c.

Sterilize at 15 pounds' pressure for fifteen minutes; or, better, steam twenty minutes for three consecutive days.

Winogradsky, S., Centbl. Bakt. (etc.), Abt. 2, Bd. 9, p. 49, 1902.

B. *Symbiotic Nitrogen-fixing Bacteria*

Medium 42

Mannit Solution

Mannit solution same as No. 39 with an excess of calcium carbonate removed.

Medium 43

Soil Extract

Soil extract	100.0 c.c.
Dibasic potassium phosphate (K_2HPO_4)	0.5 gm.
Mannit ($C_6H_8(OH)_6$)	10.0 gm.
Distilled water	900.0 c.c.

See note, page 95.

Medium 44

Saccharose Solution

Saccharose ($C_{12}H_{22}O_{11}$)	10.0 gm.
Monobasic potassium phosphate (KH_2PO_4)	1.0 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	0.2 gm.
Distilled water	1000.0 c.c.

Medium 45

Maltose Solution

Maltose ($C_{12}H_{22}O_{11} + H_2O$)	10.0 gm.
Monobasic potassium phosphate (KH_2PO_4)	1.0 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	0.1 gm.
Sodium chlorid (NaCl)	trace
Ferrous sulphate ($FeSO_4 + 7H_2O$)	trace
Calcium chlorid ($CaCl_2$) fused	trace
Distilled water	1000.0 c.c.

In order to prepare a solid medium, add 15 grams of agar to each liter of the above solutions.

Medium 46

Bean-extract Caffein Agar

Agar	15 gm.
Caffein ($C_8H_{10}N_4O_2 + H_2O$)	2 gm.
Dextrose ($C_6H_{12}O_6$)	20 gm.
Bean extract	1000 c.c.

Note.—Bean extract: Add to 100 grams of powdered bean seed in a mortar 100 c.c. of N/1 KOH. Allow this to stand a few minutes, then add water sufficient to make 5 liters. This should stand twenty-four hours. Siphon off the clear liquid, neutralize with phosphoric acid (H_3PO_4 + aq.), and make the volume up to 5 liters.

Zipfel, H., Centbl. Bakt. (etc.), Abt. 2, Bd. 32, pp. 107–131, 1911.

Medium 47

Peptone Saccharose Solution

Monobasic potassium phosphate (KH_2PO_4).....	2.0 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$).....	0.1 gm.
Peptone.....	1.0 gm.
Saccharose ($C_{12}H_{22}O_{11}$).....	20.0 gm.
Distilled water.....	1000.0 c.c.

Buchanan, R. E., Centbl. Bakt. (etc.), Abt. 2, Bd. 22, p. 392, 1909.

CELLULOSE DESTROYING ORGANISMS

(*Anaërobic*)

Medium 48

Solution for Cellulose Fermentation

Dibasic potassium phosphate (K_2HPO_4).....	1.0 gm.
Ammonium sulphate ($(NH_4)_2SO_4$).....	1.0 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$).....	0.5 gm.
Calcium carbonate ($CaCO_3$).....	2.0 gm.
Sodium chlorid (NaCl).....	trace
Distilled water.....	1000.0 c.c.

Fill large test-tubes about half-full. Add strips of filter-paper.

Omelianski, W., Centbl. Bakt. (etc.), Abt. 2, Bd. 8, p. 226, 1902.

(*Aërobic*)

Medium 49

Solution for Cellulose Fermentation

Dibasic potassium phosphate (K_2HPO_4)	0.5 gm.
Ammonium chlorid (NH_4Cl)	1.0 gm.
Calcium carbonate ($CaCO_3$)	10.0 gm.
Tap-water	1000.0 c.c.

Prepare in shallow layers (100-c.c. portions in 750-c.c. Erlenmeyer flasks) and add one sheet of filter-paper about 10 cm. in diameter to each culture.

Iterson, C. V., Centbl. Bakt. (etc.), Abt. 2, Bd. 11, p. 693, 1904.

Medium 50

Solution for Cellulose Fermentation

Dibasic potassium phosphate (K_2HPO_4)	1 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	1 gm.
Sodium carbonate (Na_2CO_3), anhydrous	1 gm.
Ammonium sulphate ($(NH_4)_2SO_4$)	2 gm.
Calcium carbonate ($CaCO_3$)	2 gm.
Tap-water	1000 c.c.

Fill large test-tubes about half-full of the above medium, or put 150-c.c. portions into 300-c.c. Erlenmeyer flasks. Immerse in the liquid of the test-tubes one or two strips of filter-paper. In the Erlenmeyer flasks use a single sheet of filter-paper of such a size that when dropped into the liquid it will nearly cover the bottom of the flask.

McBeth and Scales, Bul. 266, United States Dept. Agr. Bur. Plant Indus., p. 26, 1913.

Medium 51

Solution for Cellulose-fermenting Molds

Rye bread or bran	10.0 gm.
Dibasic potassium phosphate (K_2HPO_4)	0.5 gm.
Dextrose ($C_6H_{12}O_6$)	2.0 gm.
Distilled water	1000.0 c.c.

The rye bread or bran and water are boiled together for thirty minutes. The mixture is filtered, the loss in weight restored, and the phosphate and dextrose added.

Medium 52

Cellulose Agar

Agar (washed)	15.0 gm.
Cellulose	2.5 gm.
Dibasic potassium phosphate (K_2HPO_4)	0.2 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	0.2 gm.
Potassium carbonate (K_2CO_3)	0.4 gm.
Calcium chlorid ($CaCl_2$) fused	0.02 gm.
Ferric sulphate ($Fe_2(SO_4)_3$)	0.02 gm.
Sodium chlorid (NaCl)	0.02 gm.
Peptone	0.50 gm.
Distilled water	1000.0 c.c.

Dissolve the peptone in 40 c.c. of distilled water and filter. Dissolve the salts in the filtrate and make volume equal to 100 c.c. Add 400 c.c. of the aqueous cellulose suspension and 3 per cent. of aqueous washed agar. Tube in 15-c.c. portions.

Note.—Hydrated cellulose may be prepared as follows: To 100 c.c. of concentrated sulphuric acid in a 2-liter flask add 60 c.c. of water. When cooled to 60° C. add 5 grams of moist filter-paper. Shake this mass violently until the cellulose is dissolved. Now fill the flask with water containing

crushed ice. Transfer to a filter and wash until all traces of the acid are removed. When the volume of the filtrate is reduced to about 200 c.c., punch a hole in the filter; wash filtrate into a flask. Make volume up to 800 c.c.

Scales, F. M., Centbl. Bakt. (etc.), Abt. 2, Bd. 44, p. 661, 1915.

Medium 53

Cellulose Agar

(*a*) Agar	10 gm.
Dibasic potassium phosphate (K_2HPO_4)	1 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	1 gm.
Sodium chlorid (NaCl)	1 gm.
Ammonium sulphate ($(NH_4)_2SO_4$)	2 gm.
Calcium carbonate ($CaCO_3$)	2 gm.
Tap-water	500 c.c.
(*b*) Cellulose solution	500 c.c.

1. Pour 1000 c.c. of ammonium hydroxid, sp. gr. 0.90, into a glass-stoppered bottle; add 250 c.c. of distilled water and 75 grams of pure copper carbonate; shake the solution vigorously until all the copper is dissolved. (About ten to fifteen minutes are ordinarily required.)

2. To the copper-ammonium solution add 15 grams of high-grade sheet filter-paper; shake vigorously at intervals of ten minutes for one-half hour. Examine the solution carefully to see that the paper is completely dissolved. If any particles of paper remain in the solution, the shaking must be continued until the solution is perfectly clear. Dilute 250 c.c. of the amonium-copper-cellulose solution to 10 liters with tap-water; add slowly, with frequent shaking, a weak hydrochloric acid solution prepared by adding 500 c.c. of concentrated acid to 10 liters of tap-water. Continue the addition of the acid until the blue

color disappears; add a slight excess of acid, shake thoroughly, and allow to stand a few minutes. The finely precipitated cellulose will rise to the top, due to the large quantity of free hydrogen liberated in the precipitation process. Shake the solution vigorously at intervals of a few minutes to dislodge the hydrogen. As soon as the free hydrogen has escaped the cellulose will settle rapidly.

3. Wash through repeated changes of water untill free from copper and chlorin. After the washing is complete, bring the cellulose in the solution up to 0.5 per cent. by allowing to settle a few days, and siphoning off the clear solution or by evaporating. Add the nutrient salts desired, together with 1 per cent. of thoroughly washed agar; heat in autoclave or boil until the agar is dissolved; tube and sterilize in the usual way.

McBeth, I. G., Soil Science, vol. i, No. 5, pp. 438, 439, 1916.

Medium 54

Starch Agar

(*a*) Agar..... 10 gm.
(Salts the same as for Cellulose Agar, Medium 52.)
Tap-water..... 500 c.c.

(*b*) Starch solution..... 500 c.c.

To 10 grams of potato starch suspended in a little cold water add 800 c.c. of boiling water. Concentrate by boiling to 500 c.c. This breaks up the starch grains and should give a nearly transparent starch solution.

SULPHUR ORGANISMS

Reduction and Oxidation

Medium 55

Solution for Sulphate Reduction

Dibasic potassium phosphate (K_2HPO_4)	0.5 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	1.0 gm.
Ferrous sulphate ($FeSO_4 + 7H_2O$)	trace
Asparagin ($C_4H_8N_2O_3 + H_2O$)	1.0 gm.
Sodium lactate ($NaC_3H_5O_3$)	5.0 gm.
Tap-water	1000.0 c.c.

Van Delden, Centbl. Bakt. (etc.), Abt. 2, Bd. 11, p. 88, 1904.

Medium 56

Gelatin for Sulphate Reduction

Gelatin	120.0 to 150.0 gm.
Dibasic potassium phosphate (K_2HPO_4)	0.5 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	1.0 gm.
Iron-ammonium sulphate ($FeSO_4(NH_4)_2(SO_4) + 6H_2O$)	trace
Asparagin ($C_4H_8N_2O_3 + H_2O$)	1.0 gm.
Sodium lactate ($NaC_3H_5O_3$)	5.0 gm.
Distilled water	1000.0 c.c.

Sterilize in the autoclave at 10 pounds' pressure for fifteen minutes. Cool in ice-water.

Medium 57

Solution for Sulphate Reduction

Dibasic potassium phosphate (K_2HPO_4)	0.5 gm.
Sodium lactate ($NaC_3H_5O_3$)	5.0 gm.
Ammonium sulphate ($(NH_4)_2SO_4$)	2.0 gm.
Ferrous sulphate ($FeSO_4 + 7H_2O$)	trace
Tap-water	1000.0 c.c.

Medium 58

Sulphate Reduction

Iron lactate ($Fe(C_3H_5O_3)_2 + 3H_2O$)	5.0 gm.
Ammonium sulphate ($(NH_4)_2SO_4$)	2.0 gm.
Dibasic potassium phosphate (K_2HPO_4)	0.5 gm.
Water	1000.0 c.c.

To prepare a solid medium add the above ingredients to 15 per cent. gelatin. Heat the medium in a steamer until the precipitate has settled, and filter. Sterilize at a low temperature, about 10 pounds' pressure for fifteen minutes, or in the steamer for twenty minutes for three consecutive days.

Medium 59

Solution for Hydrogen Sulphid Formation from Protein and from Sulphur

(*a*) Iron ammonium-sulphate ($FeSO_4(NH_4)_2(SO_4) + 6H_2O$)	1 gm.
Bouillon	1000 c.c.
(*b*) Iron-ammonium sulphate ($FeSO_4(NH_4)_2(SO_4) + 6H_2O$)	1 gm.
Sulphur flowers (S)	1 gm.
Bouillon	1000 c.c.

In the presence of the proper organisms, hydrogen sulphid formation will be noted in solution (*b*) long after solution (*a*) has been reduced.

Beijerinck, W. M., Centbl. Bakt. (etc.), Abt. 2, Bd. 1, p. 5, 1895.

Medium 60

Solution for the Oxidation of Thiosulphates

Sodium thiosulphate ($Na_2S_2O_3 + 5H_2O$)	5.0 gm.
Sodium hydrogen carbonate ($NaHCO_3$)	1.0 gm.
Dibasic potassium phosphate (K_2HPO_4)	0.2 gm.
Ammonium chlorid (NH_4Cl)	0.1 gm.
Magnesium chlorid ($MgCl_2 + 6H_2O$)	0.1 gm.
Tap-water	1000.0 c.c.

Nathansohn, Mitt. a. d. zoölog. Station Neapel, Bd. 15, p. 655, 1902.
Beijerinck, W. M., Centbl. Bakt. (etc.), Abt. 2, Bd. 11, pp. 594–597, 1904.

IRON ORGANISMS

Medium 61

Solution for Thread Bacteria

Potassium acetate ($KC_2H_3O_2$)	0.5 gm.
Ferrous carbonate ($FeCO_3$)	0.5 gm.
Tap-water	1000.0 c.c.

Medium 62

Solution for Thread Bacteria

Ferrous ammonium citrate (about 16 per cent. Fe)	0.5 gm.
Tap-water	1000.0 c.c.

Medium 63

Solution for Isolating Chlamydothrix

(*a*) Agar	10.0 gm.
Manganese peptone (4 per cent. Mn_2O_3)	0.5 gm.
Tap-water	1000.0 c.c.
(*b*) Gelatin	100.0 gm.
Manganese peptone (4 per cent. Mn_2O_3)	0.25 gm.
Peat extract	1000.0 c.c.

Make the reaction slightly alkaline with normal potassium hydroxid.

Medium 64

Agar for Isolating Iron-precipitating Bacteria

Ferrous ammonium citrate (about 16 per cent. Fe)	0.5 gm.
Heyden-Nährstoff agar (see page 93)	1000.0 c.c.

Medium 65

Solution for Testing the Effect of Bacteria on Insoluble Phosphates

Asparagin ($C_4H_8N_2O_3 + H_2O$)	5.0 gm.
Sodium chlorid (NaCl)	1.0 gm.
Potassium sulphate (K_2SO_4)	1.0 gm.
Ferrous sulphate ($FeSO_4 + 7H_2O$)	0.01 gm.
Bone-meal	4.0 gm.
Distilled water	1000.0 c.c.

The bone-meal should be passed through a fine sieve and thoroughly washed. In order to secure the largest amount of soluble phosphoric acid the cultures should be incubated for sixty to ninety days.

Sackett, Patton and Brown, Bul. 43, Mich. Agr. Exp. Sta., 1908.

YEASTS

Medium 66

Solution for Yeasts

Dibasic potassium phosphate (K_2HPO_4)	0.75 gm.
Ammonium sulphate ($(NH_4)_2SO_4$)	5.0 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	0.10 gm.
Tartaric acid ($C_4H_6O_6$)	1.0 gm.
Dextrose ($C_6H_{12}O_6$)	100.0 gm.
Distilled water	1000.0 c.c.

Medium 67

Raisin Extract

Raisins	375 gm.
Ammonium chlorid (NH_4Cl)	2 gm.
Distilled water	1000 c.c.

Allow the raisins to stand in 1 liter of water for one to two days. Mash, add the ammonium chlorid, cook in the steamer for thirty minutes, and filter.

Medium 68

Solution of Yeast-water

Yeast cells	250 gm.
Distilled water	1000 c.c.

Take 250 grams of pressed yeast or 500 c.c. of washed yeast; steam in 1 liter of water for one hour. Filter while warm and steam again for thirty minutes. Make the reaction neutral to phenolphthalein, filter, and sterilize in the steamer for three successive days.

Dextrose-yeast-water may be prepared by dissolving 10 per cent. of dextrose in the yeast-water.

FUNGI

Medium 69

Solution for Fungi

Ammonium nitrate (NH_4NO_3)	10.0 gm.
Dibasic potassium phosphate (K_2HPO_4)	5.0 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	2.5 gm.
Ferrous chlorid ($FeCl_2$ + aq.)	trace
Saccharose ($C_{12}H_{22}O_{11}$)	50.0 gm.
Distilled water	1000.0 c.c.

Medium 70

Agar for Soil Fungi

Agar	15.0 gm.
Dibasic potassium phosphate (K_2HPO_4)	0.5 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	0.2 gm.
Dextrose ($C_6H_{12}O_6$)	10.0 gm.
Soil extract	200.0 c.c.
Water	800.0 c.c.

Jensen, C. N., Bul. 315, Cornell Agr. Exp. Sta., p. 430, 1912.

Medium 71

Solution for Fungi

Dibasic potassium phosphate (K_2HPO_4)	1.0 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	0.5 gm.
Potassium chlorid (KCl)	0.5 gm.
Ferrous sulphate ($FeSO_4 + 7H_2O$)	0.01 gm.
Sodium nitrate ($NaNO_3$)	2.0 gm.
Cane-sugar ($C_{12}H_{22}O_{11}$)	30.0 gm.
Distilled water	1000.0 c.c.

Medium 72

Potato Agar

Potato	200 gm.
Agar	30 gm.
Dextrose ($C_6H_{12}O_6$)	20 gm.
Distilled water	1000 c.c.

Peel and slice 200 grams of potatoes. Cook in 1000 c.c. water for one hour in the steamer. Strain or decant the clear liquid and restore it to original volume. Add 20 grams glucose and 30 grams agar. Heat in steamer until the agar is dissolved. Filter through cotton filter.

Medium 73

Clover Agar

Clover (green)	500.0 gm.
Agar	25.0 gm.
Saccharose ($C_{12}H_{22}O_{11}$)	2.0 gm.
Potassium nitrate (KNO_3)	0.5 gm.
Tap-water	1000.0 c.c.

Extract the clover tissue by heating for one hour in the steamer; filter and add the other ingredients. Add 1 to 2 drops of N/10 hydrochloric acid to each tube of the medium just before pouring plates.

ACTINOMYCETES

Medium 74

Solution for Actinomycetes

Dibasic potassium phosphate (K_2HPO_4)	0.5 gm.
Potassium nitrate (KNO_3)	3.0 gm.
Calcium malate ($CaC_4H_4O_6)_2 + 6H_2O$)	10.0 gm.
Tap-water	1000.0 c.c.

Krainsky, A., Centbl. Bakt. (etc.), Abt. 2, Bd. 41, pp. 649–688, 1914.

Medium 75

Agar for Actinomycetes

Agar	15.0 gm.
Glycerin	10.0 gm.
Sodium asparaginate ($NaC_4H_6NO_4 + H_2O$)	1.0 gm.
Glucose ($C_6H_{12}O_6$)	1.0 gm.
Ammonium hydrogen phosphate ($NH_4H_2PO_4$)	1.5 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	0.2 gm.
Calcium chlorid ($CaCl_2$) fused	0.1 gm.
Potassium chlorid (KCl)	0.1 gm.
Ferric chlorid ($FeCl_3 + 6H_2O$)	trace
Distilled water	1000.0 c.c.

Conn, H. J., Jour. Bact., vol. i, p. 198, 1916.

Medium 76

Solution for Actinomycetes

Same as No. 71.

ALGÆ

Medium 77

Solution for Algæ

Ammonium nitrate (NH_4NO_3)	0.5 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	0.2 gm.
Calcium chlorid ($CaCl_2$) fused	0.1 gm.
Ferrous sulphate ($FeSO_4 + 7H_2O$)	0.01 gm.
Dibasic potassium phosphate (K_2HPO_4)	0.2 gm.
Distilled water	1000.0 c.c.

For a solid medium add 2 per cent. of washed agar. Let the agar stand in a weak solution of alkali for several days, then wash.

Beijerinck, W. M., Centbl. Bakt. (etc.), Abt. 2, Bd. 4, p. 785, 1898.

Medium 78

Solution for Algæ

Calcium nitrate ($Ca(NO_3)_2 + 4H_2O$)	1.65 gm.
Potassium chlorid (KCl)	0.50 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	0.50 gm.
Monobasic potassium phosphate (KH_2PO_4)	0.50 gm.
Ferric chlorid ($FeCl_3 + 6H_2O$)	trace
Distilled water	1000.0 c.c.

For a solid medium add 2 per cent. of washed agar.

HIGHER PLANTS

Medium 79

Solution for Growing Higher Plants

(*a*)	Calcium nitrate ($Ca(NO_3)_2 + 4H_2O$).....	100 gm.
	Potassium nitrate (KNO_3).....	25 gm.
	Sodium chlorid (NaCl).....	15 gm.
	Distilled water.....	1000 c.c.
(*b*)	Monopotassium phosphate (KH_2PO_4).....	25 gm.
	Distilled water.....	1000 c.c.
(*c*)	Magnesium sulphate ($MgSO_4 + 7H_2O$).....	50 gm.
	Distilled water.....	1000 c.c.
(*d*)	Ferric chlorid ($FeCl_3 + 6H_2O$).....	5 gm.
	Distilled water.....	250 c.c.

Take 10-c.c. portions of solutions (*a*), (*b*), and (*c*) to 1000 c.c. of water. Add 1 to 2 drops of solution (*d*).

Tollens, B., Jour. f. Landw., Bd. 30, pp. 537–540, 1882.

Medium 80

Solution for Growing Higher Plants

(*a*)	Ammonium nitrate (NH_4NO_3).....	32.0 gm.
	Distilled water.....	1000.0 c.c.
(*b*)	Monocalcium phosphate ($CaH_4(PO_4)_2$).....	10.0 gm.
	Distilled water.....	1000.0 c.c.
(*c*)	Potassium sulphate (K_2SO_4).....	20.0 gm.
	Distilled water.....	1000.0 c.c.
(*d*)	Magnesium sulphate ($MgSO_4 + 7H_2O$).....	8.0 gm.
	Distilled water.....	1000.0 c.c.
(*e*)	Ferric chlorid ($FeCl_3 + 6H_2O$).....	0.1 gm.
	Distilled water.....	250.0 c.c.

Solutions should be prepared with ammonia-free water and chemically pure salts.

Dilute 10-c.c. portions of (*a*), (*b*), (*c*), and (*d*) and 1 c.c. of (*e*) in 1000 c.c. of water. If a nitrogen-free medium is desired, omit (*a*). Plant food solutions should be renewed at regular intervals of about one week each.

Hopkins and Pettit, Soil Fertility Laboratory Manual, p. 22, 1910.

Medium 81

Solution for Higher Plants

Potassium nitrate (KNO_3)	1.00 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	0.25 gm.
Calcium sulphate ($CaSO_4 + 2H_2O$)	0.25 gm.
Tricalcium phosphate ($Ca_3(PO_4)_2$)	0.25 gm.
Ferrous phosphate ($Fe_3(PO_4)_2$)	0.25 gm.
Distilled water	1000.00 c.c.

This solution is supposed to prevent the growth of algæ.

Crone, G., Stizungsber-Niederrhein. Gesell. Nat. und Heilkunde, Bonn., pp. 167–173, 1902.

Medium 82

Soft Agar for Plants

For growing plants 0.75 per cent. of agar in mannit solution (see page 108) is very satisfactory.

Medium 83

Solution for Higher Plants

(*a*) Monobasic potassium phosphate (KH_2PO_4)	122.5 gm.
Distilled water	1000.0 c.c.

(*b*) Calcium nitrate ($Ca(NO_3)_2$)	42.7 gm.
Distilled water	1000.0 c.c.
(*c*) Magnesium sulphate ($MgSO_4 + 7H_2O$)	90.2 gm.
Distilled water	1000.0 c.c.
(*d*) Ferric phosphate ($FePO_4 + 4H_2O$)	2.2 gm.
Distilled water	1000.0 c.c.

To prepare the complete nutrient solution, take 20-c.c. portions of (*a*), (*b*), and (*c*) and dilute to 1 liter; now add 0.5 c.c. of solution (*d*).

Shive, J. W., Physiological Researches, Vol. I, No. 7, Johns Hopkins University, pp. 327–397, 1915.

Medium 84

Solution Similar to Sea-water

Sodium chlorid (NaCl)	26.0 gm.
Magnesium chlorid ($MgCl_2 + 6H_2O$)	3.7 gm.
Potassium chlorid (KCl)	1.0 gm.
Magnesium sulphate ($MgSO_4 + 7H_2O$)	1.7 gm.
Calcium sulphate ($CaSO_4 + 2H_2O$)	1.0 gm.
Distilled water	1000.0 c.c.

This solution is recommended for the cultivation of organisms accustomed to sea-water.

Medium 85

Agar for Preserving Plate Cultures

Washed agar	20 gm.
Glycerin ($C_3H_5(OH)_3$)	500 c.c.
Distilled water	500 c.c.

Dissolve the agar in the water by heating in a steamer, add the glycerin, and filter through glass wool.

SECTION VIII

PREPARATION OF STAINS

PREPARE saturated alcoholic solutions of methylene-blue, gentian-violet, and fuchsin.

1. Place the dye in a large test-tube (about one-fourth full) and fill with 95 per cent. alcohol.

2. Let stand twenty-four hours and shake from time to time.

(1) *Löffler's Methylene-blue:*

Saturated alcoholic solution of methylene-blue	30 c.c.
Potassium hydroxid in distilled water.(1 : 10,000)	70 c.c.

(2) *Gentian-violet (Aqueous):*

Saturated alcoholic solution of gentian-violet	5 c.c.
Distilled water	95 c.c.

(3) *Carbol-fuchsin:*

Saturated alcoholic solution of fuchsin	10 c.c.
Aqueous solution of carbolic acid (5 per cent.)	90 c.c.

(4) *Gram's Iodin Solution:*

Metallic iodin	1 gm.
Potassium iodid	2 gm.
Water	300 c.c.

Dissolve in a few cubic centimeters of water. When in solution bring volume to 300 c.c.

(5) *Meissner's Solution:*

Metallic iodin	7 gm.
Potassium iodid	20 gm.
Water	100 c.c.

(6) *Aniline Oil Gentian-violet:*

Saturated alcoholic solution of gentian-violet	10 c.c.
Aniline oil in water	30 c.c.

Note.—Prepare aniline water by shaking 2 c.c. of aniline oil with 100 c.c. of water and filter until clear.

Directions for the Use of Stains

1. Take a clean cover-glass or a slide, flame, and add a loopful of sterile water.

2. With a platinum needle remove a small quantity of the bacterial colony. Do not take too much growth on the needle. Mix this thoroughly with the water and spread over the cover-glass. If the cover-glass is not clean, the water will collect in small drops.

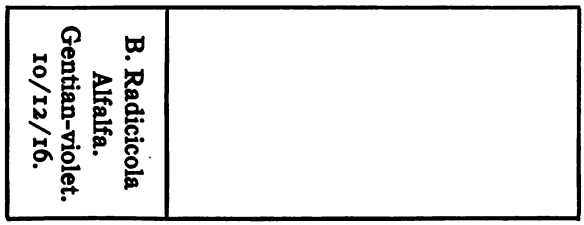
3. Allow to dry by passing back and forth high above the flame, and finally pass rapidly two or three times through the flame. This should fix the bacteria to the cover-glass and precipitate albuminous substances.

4. Flood the cover-glass with stain and let stand for ten to thirty seconds. In order to secure a deeper stain, warm gently.

5. Wash in water until the mount is clear.

6. For a temporary mount, invert the wet cover-glass on a slide, blot with filter-paper, and examine under microscope. For a permanent mount, dry the cover-glass above the flame and mount in Canada balsam or euparal.

7. The mount should be labeled as follows: Name of organism, stain, and date:



1. **Gram's Stain:**

(*a*) Spread an even, thin film.
(*b*) Fix in the flame.
(*c*) Apply aniline oil gentian-violet for two to five minutes.
(*d*) Wash in water.
(*e*) Apply Gram's iodin for one minute, or until the preparation has a coffee color.
(*f*) Wash in water.
(*g*) Decolorize with 95 per cent. alcohol until no more violet color streams out.
(*h*) Wash in water.

2. **Endospore Stain (Double):**

(*a*) Spread thin film.
(*b*) Fix by passing through flame three times.
(*c*) Stain with hot carbol-fuchsin five minutes (do not boil).
(*d*) Clean under side of slide with 2½ per cent. acetic acid.
(*e*) Decolorize the smear with 2½ per cent. acetic acid until the pink color is *nearly* removed from film.

(*f*) Wash thoroughly with distilled water.
(*g*) Dry and blot.
(*h*) Counterstain with Löffler's methylene-blue for *ten seconds.*
(*i*) Wash in water, mount, and examine.

3. **Capsule Stain:**

(*a*) Spread film *without* the *use of water.*
(*b*) Air dry.
(*c*) Fix by flaming.
(*d*) Apply glacial acetic acid, drain immediately (do not wash in water).
(*e*) Wash off acid with carbol-fuchsin three times, as rapidly as possible.
(*f*) Wash in 1 per cent. salt solution.
(*g*) Mount in the salt solution.

4. **Flagella Stain** (*Preparation of Culture*)*:*

(*a*) Transfer cultures twice each day for five days; in the morning into bouillon, and in the evening on to fresh slopes of bouillon agar.
(*b*) Carefully inoculate a tube of sterile city water. (Slant the tube and make the inoculation at the base. Then gently raise tube to upright position.) Incubate for one hour at 37° C.
(*c*) Make smears from the top of the water culture, using the greatest care in each step to prevent the breaking of the flagella.
(*d*) Air dry.
(*e*) Flame *once only.*

5. **Löffler's Flagella Stain:**

(*a*) *Preparation of the Mordant.*—Dissolve 2 grams of desiccated tannic acid in 15 c.c. of distilled water (heat gently), and add 5 c.c. of a saturated solution of ferrous sulphate, 1 c.c. of an alcoholic solution of fuchsin (1 gram of fuchsin, 25 c.c. warm absolute alcohol), 1 c.c. of 1 per cent. sodium hydrate solution. Filter.

(*a*) *Preparation of Stain.*—One part of a saturated alcoholic solution of fuchsin to $4\frac{1}{2}$ parts of a 5 per cent. solution of carbolic acid.

(*b*) Filter.

(*a*) *Method of Staining.*—Mordant one minute.

(*b*) Wash in distilled water. (Have water in wide-mouthed beaker.)

(*c*) Stain with carbol-fuchsin one-half minute (heat gently).

(*d*) Wash in water.

(*e*) Dry thoroughly.

(*f*) Treat with xylol.

(*g*) Mount in balsam.

6. **Zettnow's Flagella Stain:**

(*a*) Solution (1): Dissolve 2 grams of tartar emetic ($2K(SbO)C_4H_4O_6 + H_2O$) in 40 c.c. of water.

(*b*) Solution (2): Dissolve 10 grams of tannic acid ($C_{14}H_{10}O_9$) in 200 c.c. of water.

(*c*) Warm solution (2) to 50° to 60° C., add 30 c.c. of the tartar emetic solution (1). The turbidity of the mordant should entirely clear up on heating.

(*d*) Next dissolve 1 gram silver sulphate in 250 c.c. distilled water.

(*e*) Of this solution take 50 c.c., and add to it, drop by drop, ethylamin (33 per cent. solution) until the yellowish-brown precipitate which forms at first is entirely dissolved and the fluid is clear. Only a few drops are required.

(*f*) Float the cover-slips in a little mordant contained in a Petri dish which is heated over a water-bath for five minutes.

(*g*) Take the dish off the water-bath, and as soon as the preparation becomes slightly opalescent, wash thoroughly in distilled water.

(*h*) Then heat a few drops of the ethylamin-silver sulphate solution upon the mordanted cover preparation until it just steams and the margin appears black.

(*i*) Wash and mount in balsam.

SECTION IX

PREPARATION OF REAGENTS AND QUALITATIVE METHODS OF ANALYSIS

Pyrogallic Acid for Absorbing Oxygen.—For every 100 c.c. of air space take 1 gram of pyrogallic acid and 10 c.c. of a 10 per cent. solution of sodium or potassium hydroxid.

Note.—To Prepare an Anaërobic Jar.—Cover the bottom of an anaërobic jar with ½-inch layer of pyrogallic acid. Fit the cover tightly to the jar with vaselin and draw out the air with a suction-pump, and when there is a good vacuum, run in 75 to 100 c.c. alkali.

(1) **Phenolphthalein:**

Phenolphthalein	10 gm.
Alcohol (86 per cent.)	1000 c.c.

Dissolve the phenolphthalein in the alcohol and neutralize with sodium hydroxid until faintly pink.

(2) **Methyl-orange:**

Methyl-orange	0.2 gm.
Distilled water	1000.0 c.c.

Dissolve the solid methyl-orange in hot water, allow to cool, and if a deposit forms, filter. If the sodium salt is used instead of the acid, take 0.22 gram to 1 liter of water. Add 0.67 c.c. of normal hydrochloric acid, let stand, and filter.

(3) **Methyl-red:**

Methyl-red	2 gm.
Alcohol (95 per cent.)	1000 c.c.

Dissolve the methyl-red in alcohol and filter.

(4) **Cochineal:**

Cochineal (pulverized)	6 gm.
Alcohol (95 per cent.)	50 c.c.
Distilled water	200 c.c.

Shake the cochineal in the mixture of water and alcohol. Allow to stand for two days at room temperature. Filter until clear. The color of this solution should be a deep ruby red; in the presence of alkali a violet color, and in the presence of acid a yellowish-red color.

(5) **Preparation of Standard Solution of Sulphuric Acid.**—A normal solution of sulphuric acid is one-half the molecular weight of H_2SO_4 in grams, diluted to 1 liter with distilled water. Since the molecular weight of sulphuric acid is (2+32+64) 98, then 49 grams, one-half of 98, is the amount necessary for each liter.

1. In order to secure 49 grams of H_2SO_4, it requires 49 divided by 1.80, or 27.2 c.c. of chemically pure acid. To be sure that sufficient acid has been used, measure out about 27.5 c.c. of acid.

2. Place in 1000-c.c. graduated flask, make up to 1000 c.c., and mix carefully.

3. From this mixture remove 10-c.c. portions, accurately measured in a 10-c.c. pipet, and place in weighing bottles which have been thoroughly cleaned, dried in an oven, cooled, and weighed.

4. One c.c. of chemically pure ammonia is added to each weighing bottle to neutralize the sulphuric acid.

5. The water and excess of ammonia is then evaporated in an oven at 100° C. and the ammonium sulphate remains behind. If the chemicals are pure, 1000 c.c. of the solution should give 49 grams of sulphuric acid. In 10 c.c. of the solution there should be 0.49 gram of H_2SO_4.

$$H_2SO_4 : (NH_4)_2SO_4 :: 98 : 132$$
$$49 \quad : \quad x \quad :: 98 : 132$$
$$x = 0.66$$

If the solution is exactly normal, there should be 0.6600 gram of $(NH_4)_2SO_4$ formed from 10 c.c. In case the amount of $(NH_4)_2SO_4$ formed is too great, its factor is determined by dividing 0.6600 into the weight of ammonium sulphate found. If, for instance, the weight of ammonium sulphate is 0.6675, the factor of the solution is 1.0113+. This means that 10 c.c. of the solution is equivalent to 10.113 c.c. of normal solution.

(6) **Nessler's Reagent for Ammonia:**

1. Dissolve 50 grams of potassium iodid in a small quantity of cold distilled water.

2. Add a saturated solution of mercuric chlorid until a slight precipitate persists.

3. Now add 400 c.c. of a 50 per cent. solution of potassium hydroxid made by dissolving the potassium hydroxid and allowing it to clarify by sedimentation before using.

4. Dilute to 1000 c.c., allow to settle for one week, and decant. This solution gives the required color with ammonia within five minutes after addition.

5. Keep the Nessler's solution in a well-stoppered bottle away from the light.

Test for Ammonia.—Add to a drop of Nessler's solution in a test plate a loopful of the solution to be tested. A deep golden-yellow color indicates the presence of ammonia.

(7) **Trommsdorf's Reagent for Nitrites:**

1. Add slowly, with constant stirring, a boiling solution of 20 grams of zinc chlorid in 100 c.c. of distilled water to a mixture of 4 grams of starch in water. Continue heating until the starch is dissolved as much as possible, and the solution is nearly clear.
2. Then dilute with water and add 2 grams zinc iodid.
3. Dilute to 1 liter and filter.
4. Store in well-stoppered bottles in the dark.

Test for Nitrites.—Place 3 drops of Trommsdorf's reagent in depression on test plate. Add 1 drop of dilute sulphuric acid (1 : 3). Remove a loopful of the solution to be tested and touch to surface of reagent. A blue color indicates the presence of nitrites.

(8) **Sulphanilic Reagent for Nitrites:**

(*a*) Sulphanilic acid	0.5 gm.
Acetic acid (33 per cent.)	150.0 c.c.
(*b*) Alpha-naphthylamin	0.1 gm.
Distilled water	20.0 c.c.
Acetic acid (33 per cent.)	150.0 c.c.

Dissolve the alpha-naphthylamin by heating in 20 c.c. of water, filter, then add the acetic acid. Combine solutions (*a*) and (*b*), and keep in a tightly stoppered bottle. This solution is sensitive to 2 parts of nitrite in 10,000,000, giving a reddish-pink color.

Griess, Ber. d. deutsch. chem. Gesell., 12, p. 426, 1879.

(9) **Diphenylamin Reagent:**

1. Dissolve 0.7 gram of diphenylamin in a mixture of 60 c.c. of concentrated sulphuric acid and 28.8 c.c. of distilled water.

2. Cool this mixture and add slowly 11.3 c.c. of concentrated hydrochloric acid (sp. gr. 1.19). After standing overnight some of the base separates, showing that the reagent is saturated.

Withers and Ray, Jour. Amer. Chem. Soc., vol. xxxiii, pp. 708–711, 1911.

Test for Nitrates.—Place 1 drop of the substance to be tested in a depression on the test plate. Add 1 drop of diphenylamin solution and allow the solutions to mix thoroughly. Then add 2 drops of concentrated sulphuric acid. A deep blue color indicates nitrates. This test cannot be made in the presence of nitrites, chloric and selenic acids, ferric chlorid, and many other oxidizing agents.

In order to detect nitrates in the presence of nitrites, add a concentrated solution of urea to a small amount of the liquid in a test-tube. Now add in the bottom of the tube (by means of a pipet) a dilute solution of sulphuric acid. This should destroy a greater part of the nitrous acid.

$$CO(NH_2)_2 + 2HNO_2 = CO_2 + 3H_2O + 2N_2$$

(10) **Brucin Reagent.**—Dissolve 1.0 gram of brucin in 10 c.c. of 50 per cent. pure concentrated sulphuric acid and make up to 100 c.c. with distilled water.

Test for Nitrates.—Place 1 drop of the substance to be tested in a depression on the test plate and add 3 drops of concentrated sulphuric acid. Now add 1 drop of brucin solution. If nitrates are present, a red color develops

quickly, which changes to orange, then slowly to lemon or yellow, and finally becomes a greenish yellow.

(11) **Phenolsulphonic Acid.**—Dissolve 25 grams of pure white phenol crystals in 150 c.c. of pure concentrated sulphuric acid, add 75 c.c. of fuming sulphuric acid (13 per cent. SO_3), stir well, and heat for two hours at about 100° C. The reagent prepared in this way should not contain any mono-acids or any tri-acids. Two c.c. of this reagent give reliable results with not more than 5 milligrams of nitrate nitrogen.

Chamot, Pratt, and Redfield, Jour. Amer. Chem. Soc., vol. xxxiii, pp. 381–384, 1911.

(12) **Fehling's Reagents:**

(*a*) Copper sulphate ($CuSO_4 + 5H_2O$)	34.639 gm.
Distilled water	500.0 c.c.
(*b*) Sodium potassium tartrate ($KNaC_4H_4O_6 + 4H_2O$)	178.0 gm.
Sodium hydroxid	50.0 gm.
Distilled water	500.0 c.c.

Pulverize the crystalline substances before attempting to dissolve.

Qualitative Test.—Mix equal quantities of (*a*) and (*b*), about 5 c.c. of each. Add an equal amount of the solution to be tested and boil. A red precipitate indicates reducing sugar.

(13) **Citric Acid Reagent:**

Mercuric sulphate solution	50 gm.
Sulphuric acid (conc.)	200 c.c.
Distilled water	1000 c.c.

Test for Citric Acid.—To a water solution of the citric acid add 2 c.c. of the mercury reagent and boil. Now add 5

to 10 drops of a potassium permanganate solution (2 grams to 1000 c.c.). In the presence of citric acid the solution becomes colorless and a white precipitate is formed.

Abderhalden, E., Handbuch der Biochemischen Arbeitsmethoden, Bd. 5, p. 409, 1913.

Tests for Indol and Skatol:

(A) 1. Add a few drops of a 5 per cent. solution of vanillin in 95 per cent. alcohol to 5 or 6 c.c. of the indol solution.

2. Make the mixture strongly acid with 3 to 4 c.c. of concentrated hydrochloric acid. A beautiful orange color denotes *indol.*

3. If skatol is present, the same reagents produce a deep violet color upon heating. These tests are very sensitive.

4. The color formed with indol is only very slightly soluble in chloroform, while the color with skatol is soluble in chloroform.

Nelson, V. E., Jour. Biol. Chem., vol. xxiv, pp. 527–532, 1916.

(B) 1. Add 1 c.c. of a 0.01 per cent. solution of potassium nitrite.

2. Now add a few drops of sulphuric acid and warm in a water-bath. In the presence of indol a pink color appears.

3. If a solution containing skatol is treated with a few drops of nitric acid and a dilute solution of potassium nitrite, a white turbidity is noted.

(14) **Millon's Reagent:**

Mercury metallic	50 gm.
Nitric acid (sp. gr. 1.42)	100 gm.

Dissolve the mercury in its weight of concentrated nitric acid and dilute with an equal volume of water. Only

freshly prepared solution should be used. Proteins when heated with Millon's reagent turn a brick red.

Biuret Reaction.—Add sodium or potassium hydroxid to a dilute sulphuric acid solution containing protein until alkaline, and a few drops of a very dilute solution of copper sulphate. The presence of protein will be marked by the gradual spreading of a reddish-violet color through the solution.

Mercuric Chlorid.—A stock solution is prepared and diluted to the desired strength.

Stock Solution: Add 1 part of mercuric chlorid to 2.5 parts of commercial hydrochloric acid (40 per cent. $HgCl_2$ in HCl).

In order to prepare a 1 : 1000 solution, the concentration commonly used for disinfecting purposes, take 2.5 c.c. of the stock solution and dilute to 1000 c.c.

Solution for Sealing Bottles.—Melt together equal parts of gutta-percha and paraffin.

Seal for Museum Jars.—A transparent seal for museum jars may be made by wetting celluloid with acetone. Cut strips or rings of celluloid a little wider than the ground-glass surface, dip in acetone, and place upon the edge of the jar. Cover before the acetone evaporates and press slightly.

Preserving Plants in Natural Colors.—Saturate with copper acetate a 50 per cent. glacial acetic acid solution in water.

Take 4 parts of water to 1 part of the stock solution. Boil the plant tissue to be preserved for five to ten minutes, or until the color becomes yellowish and then green. Wash in water and preserve in a 4 or 5 per cent. solution of formalin.

SECTION X

QUANTITATIVE METHODS OF ANALYSIS

(1) **Moisture.**—Weigh from 5 to 10 grams of soil into a glass or aluminum dish and dry at 100° C. until there is no further change in weight. About six to twelve hours are generally sufficient. Cool in a desiccator and weigh. Determine all percentages of moisture on the dry basis.

Record data as follows:

Weight of dish and moist soil .....
Weight of dish and dried soil .....
Loss .....

$$\text{Percentage of moisture} = \frac{(\text{A D S} - \text{W F S})}{\text{W F S}} \times 100$$

A. D. S. = air-dry soil.
W. F. S. = water-free soil.

(2) **Ammonia (Distillation):**

Sulphuric acid solution..... N/14
Sodium hydroxid solution..... N/14
Indicator, cochineal or methyl-red.
Magnesium oxide.

1. Transfer the culture to be analyzed to an 800-c.c. Kjeldahl or a copper flask, using about 200 c.c. of distilled water.

2. Add 5 grams of magnesium oxid and some shavings of paraffin to prevent foaming.

3. Connect to a condenser, the lower end of which is in N/14 acid.

4. Erlenmeyer flasks of good quality should be used to collect the distillate.

5. The apparatus for this work differs from the usual Kjeldahl stand in that a jet of steam is passed directly into the distilling flask. It is so arranged that the rubber stopper for the Kjeldahl flask has two holes, one for the condensing bulb and one for the steam tube. Steam is allowed to bubble slowly through the solution in the bottom of the Kjeldahl flasks. In order to hasten the analysis a very low flame should be kept under the distilling flask. The period of distillation will vary with the amount of ammonia present. As a rule, one hour is long enough to drive off all ammonia nitrogen.

6. If methyl-red is used as an indicator, the distillate should be boiled for a few minutes, cooled to 15° or 20° C., about 5 drops of methyl-red added, and the solution titrated.

7. The distillate is titrated with standard alkali, and from the cubic centimeters of standard acid neutralized by the distillate the weight of nitrogen liberated as ammonia is calculated.

(3) **Ammonia (Nesslerization).**—*Ammonia-free Water.*—This is readily prepared by adding sodium hydroxid and potassium permanganate to laboratory water and redistilling. Discard the first portion of the distillate. After about one-fourth of the water has been evaporated, the subsequent distillate will be free of ammonia.

Standard Ammonium Chlorid Solution.—Dissolve 3.82 grams of ammonium chlorid in 1000 c.c. of distilled water; dilute 10 c.c. of this to 1000 c.c. with ammonia-free water. One c.c. equals 0.01 mg. of nitrogen.

1. Prepare a series of sixteen Nessler's tubes which contain the following number of cubic centimeters of the standard ammonium chlorid solution, dilute to 50 c.c.

with ammonia-free water, namely, 0.0, 0.1, 0.3, 0.5, 0.7, 1.0, 1.4, 1.7, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, and 6.0.

2. These will contain 0.01 mg. of ammonia nitrogen for each cubic centimeter of the standard solution used.

3. Nesslerize the standards and also the distillates by adding approximately 2 c.c. of Nessler's reagent to each tube.

4. Do not stir the contents of the tubes.

5. After Nesslerizing, allow the tubes to stand for ten minutes.

6. Compare the color produced in these tubes with that in the standards by looking vertically downward through them at a white surface placed at an angle in front of a window, so as to reflect the light upward.

(4) **Nitrates (Colorimetric):**

1. Evaporate in a porcelain dish on a water-bath a convenient quantity of unknown nitrate solution, depending upon the amount of nitrate present, to dryness.

2. When evaporated, add 2 c.c. of phenoldisulphonic acid and stir with the rounded end of a glass rod for about ten minutes so as to loosen the residue.

Note.—Equations for the action of phenoldisulphonic acid on a nitrate:

$$H_2SO_4 + 2KNO_3 = 2HNO_3 + K_2SO_4$$

$$C_6H_3(OH)(SO_3H)_2 + HNO_3 = C_6H_2(OH)(SO_3)_2(NO_2) + H_2O$$

$$C_6H_3(OH)(SO_3H)_2(NO_2) + 3NH_4OH = C_6H_3(ONH_4)(SO_2ONH_4)_2NO_2 + 3H_2O$$

3. Dilute with water and add ammonia solution (strong ammonium hydroxid diluted with an equal volume of water) until alkaline; a yellow color is formed. This is then diluted to a known volume and compared with the standard.

For example, take 500 c.c. of water to 100 grams of soil,

and in order to clarify add about 2 grams of calcium oxid. To secure a fair sample, mix by rubbing in a mortar or by shaking in a wide-mouthed bottle. Filter through folded filter-paper until clear. Take a convenient volume, for example, 25 c.c., and determine the nitrate present. This is equal to 5 grams of soil. Use the colorimeter to compare the standard solution with the unknown.

Formula for calculating results:

$$X = \frac{100}{W} \cdot \frac{S}{A} \cdot d \cdot \frac{K}{U} \cdot M$$

Where X = Number of milligrams of N as NO_3 per 100 grams dry soil.
W = Weight of dry soil.
S = Cubic centimeters of water added to W.
A = Aliquot taken for evaporation.
d = Number of cubic centimeters to which A was diluted.
K = Reading on scale of standard solution.
U = Reading on scale of unknown solution.
M = Milligrams of N as NO_3 in 1 c.c. of standard solution as diluted for reading.

Standard Nitrate Solution.—Dissolve 0.722 gram of pure dry potassium nitrate in 1000 c.c. of water. Of this strong solution dilute 10 c.c. to 100 c.c., and from this take 10 c.c. for a standard. Evaporate to dryness in a porcelain dish on a water-bath and treat as described above. Make up volume to 100 c.c. Each cubic centimeter of this standard is equal to 0.001 milligram of N as nitrate, or 100 c.c. of this standard is equal to 0.1 milligram of nitrogen.

(5) **Nitrates (Reduction):**

1. Add to 250 or 500 c.c. of aqueous soil extract in an 800-c.c. Kjeldahl flask 5 c.c. of a 50 per cent. sodium hydroxid solution; partially close the mouth of the flask with a small funnel to prevent spattering and boil for half an hour.

2. Replace the water driven off in heating.

3. When cool, add 2 grams of finely divided Devarda's alloy (about 60 mesh) and connect the flask with the distilling apparatus.

4. The distillation should not be hurried.

5. Allow the solution to boil for thirty to sixty minutes.

(6) **Total Nitrogen Without Nitrates:**

Sulphuric acid..... N/14
Sodium hydroxid..... N/14
Sulphuric acid (concentrated).
Potassium or sodium sulphate.
Copper sulphate.
Pumice powder.
Sodium hydroxid (50 per cent.).

1. Place the substance to be analyzed in a Kjeldahl flask (the amount for analysis will depend on the nitrogen content); if soil is used, about 10 grams.

2. Add 5 grams of powdered potassium sulphate or sodium sulphate, 0.5 gram copper sulphate, 30 to 40 c.c. of sulphuric acid, and mix thoroughly. It is important that the substance be thoroughly moistened by the sulphuric acid before heating.

3. Place the flask on the digestion shelf under a hood and heat slowly until frothing ceases. Avoid a very high flame; do not allow the flame to touch the flask above the part occupied by the liquid. If sugar is present, for example, mannit agar culture, the acid mixture will foam very badly. In order to prevent any loss it is well to heat *very slowly* until all foaming has ceased. Sometimes this requires one hour or more.

4. Now raise the heat (avoid a very hot flame) until the acid boils rapidly.

5. Digest for thirty minutes after the acid mixture is

colorless. If sugar is absent, about two to three hours is sufficient for complete digestion.

6. In case the contents of the flask are likely to become solid before digestion is complete, cool, and add 10 c.c. more of sulphuric acid.

7. When digestion is complete, cool, and add 200 c.c. of water. Shake until the mixture is thoroughly in solution. Be sure that none of the digested material remains caked to the sides of the Kjeldahl flask.

8. Recool, add a teaspoonful of powdered pumice to prevent bumping, and shake thoroughly.

9. Add 100 c.c., or more if necessary, of a saturated sodium hydroxid solution. (The stock solution of alkali should be prepared two days or more before it is to be used in order that the sodium carbonate may precipitate out. Avoid the deposit in the bottom of the alkali.) Enough alkali should be added to make the solution react strongly alkaline. A few strips of litmus-paper may be added in order to test the reaction. The alkali should be poured *slowly* down the sides of the flask. After about half of the alkali is added, it is well to shake the solution. Now add the remaining alkali and connect at once to the condenser.

10. See that the rubber stopper fits snugly in the flask. *Now mix the contents thoroughly by shaking.*

11. Just before connecting the flask have a very low flame burning on the distillation shelf. After the alkali and acid mixture are well mixed, raise the flame.

12. The proper amount of standard acid should be measured into flasks connected to the distillation shelf prior to adding the alkali.

13. Distil slowly. After the first fifteen minutes the flame may be raised, but never so high that the distillate collects in the condensing bulbs. Generally the first two-

thirds of the original volume recovered as distillate will contain all the ammonia.

14. The distillate is now titrated with standard alkali, and from the cubic centimeters of standard acid neutralized by the distillate the weight of nitrogen liberated as ammonia is calculated.

15. This should be reported as percentage of nitrogen on the dry basis.

(7) **Total Nitrogen with Nitrates Present:**

Same as for (6); in addition:
Salicylic acid.
Sodium thiosulphate.

1. Add to the substance to be analyzed in a Kjeldahl flask 35 to 40 c.c. of sulphuric acid with salicylic acid (1 gram in 30 c.c. of sulphuric acid); shake until thoroughly mixed and allow to stand five or ten minutes, with frequent shaking.

2. Now add 5 grams of crystallized sodium thiosulphate and heat the solution gently for five minutes, then bring to boiling for five minutes; cool; add 0.5 gram of copper sulphate and boil. This reduces the danger of foaming.

3. Heat very gently until foaming ceases, then heat strongly until colorless. Continue boiling for thirty minutes after the substance is colorless. The entire process requires five to six hours.

4. Cool and add about 200 c.c. of distilled water.

5. Cool again, and add a few pieces of granulated zinc or pumice powder to keep the contents of the flask from bumping during distillation.

6. Next add 100 c.c. or more of strong soda solution sufficient to make the reaction strongly alkaline, pouring it down the sides of the flask so that it does not mix at once with the acid.

7. Connect the flask with the condenser (having prepared the acid to receive the ammonia). *Mix the contents thoroughly by shaking*, and distil until all the ammonia has passed over into the standard acid.

(8) **Humus:**

1. Extract 10 grams of air-dry soil in a Gooch crucible with 1 per cent. hydrochloric acid until the filtrate gives no precipitate with ammonium hydroxid and ammonium oxalate.

2. Wash until all the acid is removed. In the case of clay soil, the washing should be done chiefly by decantation from a cylinder or tall beaker.

3. Wash the contents of the crucible (including the asbestos filter) into a glass-stoppered cylinder, with 500 c.c. of 4 per cent. ammonium hydroxid. (Mix 300 c.c. of water with 200 c.c. of ammonia (sp. gr. .90) and add more water or ammonia until the hydrometer reads .9604, which is exactly 20 per cent. solution of ammonium hydroxid (NH_4OH). Dilute this to 4 per cent. with water.)

4. Allow to remain, with occasional shaking, for twenty-four hours. During this time the cylinder is inclined as much as possible without bringing the contents in contact with the stopper; thus allowing the soil to settle on the side of the cylinder and exposing a very large surface to the action of the ammonium hydroxid.

5. Place the cylinder in a vertical position and leave for twelve hours to allow the sediment to settle.

6. Draw off 300 c.c. of the supernatant liquid with a pipet, without stirring up the sediment, place in a stoppered 500-c.c. flask, and let stand for forty-eight hours.

7. Carefully pipet off 200 c.c. of the liquid, free of clay particles, into a 300-c.c. beaker, evaporate it on a steam-bath, and let the residue heat on the bath for two hours.

8. Dissolve out the humus with 200 c.c. of 4 per cent. ammonium hydroxid and filter through paper to separate the flocculated clay.

9. Evaporate 50 c.c. aliquots, dry at 100° C., and weigh.

10. Ignite the residue and reweigh.

11. Calculate the humus from the difference in weights between the dried and ignited residues.

12. Report as percentage of the dry soil.

(9) **Carbon Dioxid:**

1. Connect a large Erlenmeyer suction flask of about 2-liter capacity with a long glass tube by means of a 50-c.c. pipet bent as shown in Fig. 10.

2. Fill the glass cylinder about two-thirds full of glass beads and 10 c.c. of normal potassium hydroxid. The beads prevent the carbon dioxid from passing through the alkaline solution too rapidly, and afford much greater surface. The 50-c.c. pipet prevents suction of the alkali back into the flask.

3. Place 1 kilogram of soil in the Erlenmeyer flask.

4. Connect the 50-c.c. pipet to the flask and close the ends of the glass tubes by means of clamps.

5. In order to conduct the carbon dioxid into the potassium hydroxid solution draw a current of air slowly through the apparatus for ten to twenty minutes each day.

6. The current of air should be freed of carbon dioxid by passing through strong alkali or a series of soda-lime tubes. The amount of air may be determined by counting the air bubbles.

7. After the carbon dioxid has been collected in the potassium hydroxid, disconnect the suction flask and transfer the contents of the glass tower into a 500-c.c. Erlenmeyer flask by successive washings with small portions of carbon-dioxid-free water. This is easily accomplished

by inclining the end of the pipet into the flask and pouring the wash-water on to the beads. Washing should be repeated until all traces of alkali have disappeared. Place a few drops on a watch-glass containing phenolphthalein. If there is no change in color, the washing is complete.

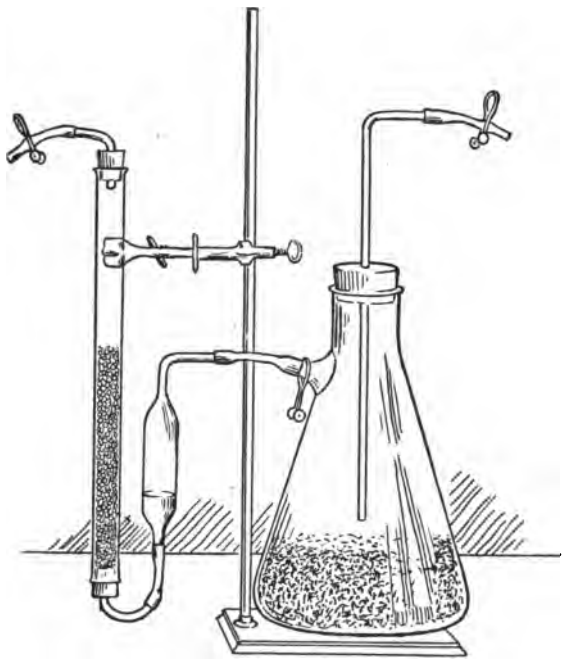


Fig. 10.—Apparatus for determining carbon dioxid.

Avoid using too large a quantity of water for each washing, otherwise the volume of liquid to titrate will be very large.

Note.—Carbon-dioxid-free water may be prepared by drawing a current of carbon-dioxid-free air through distilled water for twenty-four hours or longer.

8. Now add about 6 drops of phenolphthalein to the solution and titrate with N/2 sulphuric acid until the *pink color begins to change. Care should be taken not to overtitrate.* In order to secure the best results titrate with the tip of the buret in the solution.

9. At this point, when the carbon exists as acid carbonate, add 5 drops of methyl-orange and titrate with N/10 sulphuric acid until the color changes.

10. Prepare color standards for each indicator by adding to carbon-dioxid-free water the same amount of the indicator as used in titrating.

11. The number of cubic centimeters of N/10 sulphuric acid used during the titration with methyl-orange as an indicator, minus the blank, multiplied by 1.2, represents the weight in milligrams of carbon produced as carbon dioxid from 1 kilogram of soil.

Equation for the double titration of carbon dioxid:

$$2KOH + CO_2 = K_2CO_3 + H_2O$$

$$K_2CO_3 + H_2SO_4 = KHSO_4 + KHCO_3$$

$$KHCO_3 + H_2SO_4 = KHSO_4 + H_2CO_3 = H_2O + CO_2$$

(10) Soil Acidity:

Lead acetate paper.

Calcium chlorid solution plus zinc sulphid solution (2 per cent. of ZnS in 20 per cent. $CaCl_2 + 2H_2O$).

1. Place 10 grams of the soil to be analyzed in a 300-c.c. Erlenmeyer flask.

2. Add 50 c.c. of a mixture of 45 c.c. of water and 5 c.c. of a well-shaken suspension of zinc sulphid in calcium chlorid solution.

3. In order to remove the zinc sulphid adhering to the vessel, refill with 50 c.c. of water and add to the flask.

4. Place the flask at once over the flame and boil for one minute after violent bubbling starts.

5. Now place a strip of lead acetate paper moistened with not more than 3 drops of water over the mouth of the flask.

6. Boil for two minutes, remove, and dry the paper.

7. Compare the paper with standard color chart.

Truog, E., Bul. 249, Wis. Agr. Exp. Sta., 1915.

(11) **Reducing Sugars (Defren-O'Sullivan):**

1. Mix 15 c.c. of Fehling's copper solution (see p. 138) (*a*) with 15 c.c. of the alkaline tartrate solution (*b*) in a 300-c.c. Erlenmeyer flask, and add 50 c.c. of distilled water.

2. Place the flask and its contents in a water-bath containing boiling water and allow it to remain five minutes.

3. Then run rapidly from a buret into the hot liquor in the flask 25 c.c. of the sugar solution to be tested, which should contain not more than $\frac{1}{2}$ per cent. of reducing sugar.

4. Allow the flask to remain in the boiling water just fifteen minutes after the addition of the sugar solution, remove, and, with the aid of a vacuum, filter the contents rapidly through a porcelain Gooch crucible containing a layer of prepared asbestos fiber about $\frac{1}{8}$ inch thick, the Gooch, with the asbestos, having been previously ignited, cooled, and weighed.

5. The cuprous oxid precipitate is washed thoroughly with boiling distilled water until the water ceases to be alkaline. The asbestos used should be of the long-fibered variety and should be especially prepared as follows: Boil first with nitric acid (sp. gr. 1.05–1.10), washing out with hot water; then boil with a 25 per cent. solution of sodium hydroxid; and finally wash out the alkali with hot water. Keep the asbestos in a wide-mouthed bottle and transfer

it to the Gooch by shaking it up in the water and pouring it quickly into the crucible while under suction. The excess of fine asbestos should be poured off before adding to crucible.

6. Dry the Gooch with its contents in the oven, and finally heat to dull redness for fifteen minutes, during which the red cuprous oxid is converted into the black cupric oxid. Considerable care must be taken to avoid cracking the crucible, the heat being increased cautiously, and the operation conducted preferably in a muffle furnace.

7. After oxidation as above, the crucible is transferred to a desiccator, cooled, and quickly weighed.

8. From the milligrams of cupric oxid calculate the milligrams of dextrose according to the following table:

Leech, Food Inspection and Analysis, New York, p. 595, 1913.

Defren's Table for Dextrose, Maltose, and Lactose

Cupric oxid.	Dextrose.	Maltose.	Lactose.	Cupric oxid.	Dextrose.	Maltose.	Lactose.
Mgm.	Mgm.	Mgm.	Mgm.	Mgm.	Mgm.	Mgm.	Mgm.
30	13.2	21.7	18.8	180	80.4	131.8	114.6
40	17.6	29.0	25.2	190	84.9	139.1	121.0
50	22.1	36.2	31.5	200	89.5	146.6	127.5
60	26.5	43.5	37.8	210	94.0	154.1	134.1
70	30.9	50.8	44.1	220	98.6	161.5	140.6
80	35.4	58.1	50.5	230	103.1	169.1	147.0
90	39.9	65.5	56.8	240	107.7	176.6	153.5
100	44.4	72.8	63.2	250	112.3	184.1	160.0
110	48.9	80.1	69.5	260	116.9	191.6	166.5
120	53.3	87.4	75.9	270	121.4	199.2	173.0
130	57.8	94.8	82.4	280	126.1	206.8	179.6
140	62.2	102.1	88.7	290	130.7	214.3	186.2
150	66.8	109.5	95.2	300	135.3	221.9	192.8
160	71.3	116.9	101.7	310	139.9	229.6	199.3
170	75.8	124.4	108.2	320	144.5	237.2	205.9

(12) **Hydrogen Sulphid:**

1. Prepare the following reagents: N/100 iodin and N/100 sodium thiosulphate. Standardize the iodin against the thiosulphate solution. One c.c. of the N/100 iodin solution is equivalent to 0.17 milligram of hydrogen sulphid, using starch as an indicator.

2. Add to a large glass-stoppered bottle 10 c.c. of the iodin solution and 2 grams of potassium iodid.

$$H_2S + 2I = 2HI + S$$

3. Take 1000 c.c. of the sample to be tested, pour it into the large bottle containing the iodin, shake thoroughly, and allow to stand for some time.

4. Titrate the excess iodin with N/100 thiosulphate.

5. In the presence of large amounts of hydrogen sulphid use tenth-normal iodin instead of one-hundredth normal.

Treadwell, Analytical Chemistry, vol. ii, p. 687, 1912.

SECTION XI

SPECIAL METHODS

FOR a detailed discussion of sterilization see one of the standard works on bacteriologic technic. Here only special phases of this subject will be considered.

Seed Sterilization.—Although a great number of methods employing various agents have been recommended for removing micro-organisms from seed, only a few of the more promising ones will be given. Where it is not necessary to render the seeds free of bacteria, but merely to destroy the majority of the flora, alcohol may be used.

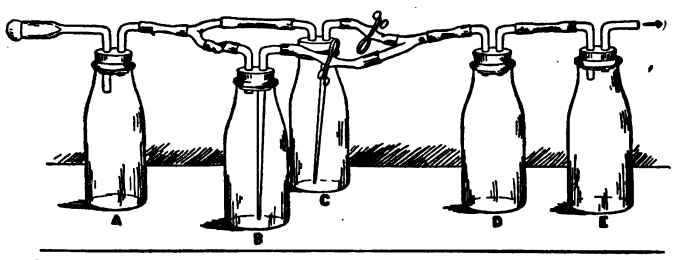


Fig. 11.—Apparatus for seed sterilization.

Among the chemicals that have proved satisfactory for sterilizing seed, mercuric chlorid, hypochlorate of lime, and silver nitrate are the most commonly used. The effectiveness of these substances depends on many factors: strength of solution, time of exposure, temperature, pressure, and nature of the seed coat.

Mercuric Chlorid in Vacuum:

1. Set up the apparatus shown in Fig 11, using heavy walled bottles (milk) and heavy steam-proof rubber connections.

2. Fill flask B with 0.25 per cent. solution of mercuric chlorid and C with distilled water.

3. After the whole apparatus is connected, the clamps between bottles B–D and C–D fastened, sterilize in the autoclave at 10 pounds' pressure for fifteen minutes.

4. Cool to 40° C., connect to a vacuum pump, and place the seed in flask D. If the seed are small, place a layer of cheese-cloth over the mouth of bottle before inserting stopper. The seed should be thoroughly clean before sterilizing. It is well to wash with 60 to 70 per cent. alcohol.

5. By means of the vacuum pump draw the mercuric chlorid from B to D; then close the screw-clamp and exhaust D for three to five minutes. This should remove the air particles from around the seed coats and allow the disinfectant to come in direct contact with the seed.

6. At the end of this time invert D and withdraw the mercuric chlorid solution. Now run in a small amount of sterile water from C, shake vigorously, empty, and repeat this process three or four times.

7. Remove some of the seed to sterile Petri dishes and pour over them a layer of bouillon agar.

8. After the agar hardens, invert and place in the incubator at 20° to 25° C. In two or three days the seed should germinate. If bacteria or molds are present, they may be readily noted on the agar.

Hutchinson and Miller, Jour. Agr. Sci., vol. iii, p. 185, 1908.

Calcium Hypochlorite:

1. Add 10 grams of commercial chlorid of lime (titrating 28 per cent. chlorin) to 140 c.c. of water.

2. Allow the mixture to settle for five or ten minutes and decant the supernatant liquid. This solution should contain about 2 per cent. of chlorin.

3. For seed sterilization the solution may be diluted or used full strength. The volume of the liquid should be about five times that of the seed.

4. Place the seed in a sterile test-tube and cover with a 1 per cent. chlorin solution (original solution diluted one-half).

5. The time required for sterilizing varies with the different seed, about six hours for alfalfa, eight hours for corn, and fifteen hours for wheat.

Wilson, J. K., Amer. Jour. Bot., vol. ii, pp. 420–427, 1915.

Silver Nitrate.—According to Schroeder the Gramineæ are not readily penetrated by silver nitrate, and withstand treatment with a 5 per cent. solution for twelve to twenty-four hours. In order to remove the silver nitrate wash thoroughly in a sodium chlorid solution and allow the seed to stand in a dilute solution of sodium chlorid for twenty-four hours.

Schroeder, H., Centbl. Bakt. (etc.), Abt. 2, Bd. 28, pp. 492–505, 1910.

Soil Sterilization.—In order to destroy all forms of microörganisms in soil a high temperature for a long period of time is required. It is impossible to sterilize soil by the methods commonly employed for culture-media. Unfortunately, the temperature required to kill bacterial spores in soil brings about other changes, chemical and physical. In some cases sterilization results in undesirable

changes; in others it seems to improve the crop-producing power of the soil.

For a discussion of this problem see the publications of Richter, Pickering, Seaver, and others.

Richter, Landw. Vers. Stat., Bd. 47, p. 269, 1896.
Pickering, Jour. Agr. Sci., vol. ii, p. 411, 1908; vol. iii, p. 32, 1908.
Seaver, Mycologia, vol. i, p. 131, 1909.
Seaver and Clark, Biochemical Bul. No. 9, p. 413, 1912.
Lyon and Bizzell, Bul. 275, Cornell Exp. Sta., 1910.
Lathrop, Bul. 89, Bur. of Soils, U. S. Dept. Agr., 1912.
Johnson, J., Science, vol. xliii, pp. 434, 435, 1916.

Method for Sterilizing Soil:

1. Small test-tubes of soil may be sterilized by heating in the autoclave for two hours on two successive days at 15 pounds' pressure.

2. When it is desirable to sterilize much larger amounts of soil, the time of heating should be increased.

3. If earthenware jars are used, the cold air in the bottom is removed very slowly, therefore it is necessary to heat for several hours.

4. A 4-liter earthenware jar of soil requires at least six hours or longer at 15 pounds' pressure to kill all forms of bacteria.

Growing Plants Free of Microörganisms.—The method to follow in growing plants free of bacteria depends largely upon two factors: the size of the plant and the time of growing period. If small plants are used—clover, alfalfa, etc.—and it is not necessary to grow to maturity, large test-tubes or glass cylinders may be used.

Kellerman, K. F., Cir. 120, U. S. Dept. Agr. Bur. Plant Ind., 1913.

In order to grow plants for a long period of time in a medium free from infection a vessel of special design is

necessary. Although there are a great number of vessels designed for this purpose, only one will be described. It is obvious from the start that apparatus of this nature must be somewhat complicated. A modification of the Schulze and Schulow methods has been found fairly satisfactory.

Apparatus:

One large Woulfe's bottle, about 3-liter capacity, with three openings.
One large 2- or 3-liter flask, Erlenmeyer form.
Two U tubes.
One cylinder constructed as shown in Fig. 13.

The apparatus is arranged according to Fig. 12.

Schulow, I., Ber. d. Deut. Bot. Gesell., Bd. 29, p. 504, 1911.
Schulze, C., Landw. Jahrb., Bd. 30, p. 327, 1901.

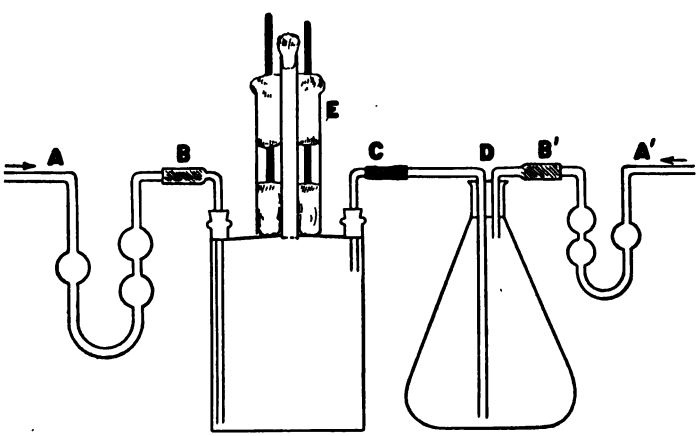


Fig. 12.—Complete apparatus for growing plants free of bacteria.

All stoppers and connecting tubes should be made of steam-resistant rubber. The U tube A and the long hard-glass tube B, enlarged at one end for sterile cotton

and stopper, serve to remove any micro-organisms from the air. If a liquid medium is used, the aëration tubes A and B may be omitted. A′ and B′ are prepared in the same way as A and B. The tubes A′, B′, D, and C are used to carry water over from the flask to Woulfe's bottle.

Prior to filling the Woulfe bottle with soil wrap some glass-wool around the end of tube B, and cover the bottom of the bottle with an inch layer of gravel. Now add the soil and raise the water content to about half-saturation. The special glass cylinder E which fits loosely around the middle neck of Woulfe's bottle consists of a large glass cylinder 4 to 5 cm. in diameter and 15 cm. in length. Within this cylinder there is a glass tube about $1\frac{1}{4}$ to $1\frac{1}{2}$ cm. in diameter and 20 cm. long, which reaches to the wire net (see Fig. 13). The top of the glass tube carries a cotton plug. Between the two cylinders there is loose cotton packing and three glass rods about 0.4 cm. in diameter and 15 cm. long (see Fig. 13).

The entire apparatus should be connected as shown in Fig. 12; the flask filled almost full of distilled water, a screw-clamp fastened between C and D in order to prevent the water in the flask from flowing over into the bottle, and sterilized for two hours at 15 pounds' steam pressure for two consecutive days. If carefully wrapped in paper, heated and cooled slowly, there is not much danger of cracking the glass. When cool, seal all stoppers with a beeswax-rosin mixture.

In view of the long time required for plant growth and the danger of infection, it is well to keep the cultures in a clean room as free from contamination as possible.

To plant, remove the cotton plug from the inner glass cylinder or tube (see Fig. 13), and by means of sterilized forceps drop the seed down the inner tube on the wire net.

As soon as the young shoots are 5 to 10 cm. long, raise the inner glass tube slightly (Fig. 13, B) and push the cotton firmly around the base of the young shoot. This should

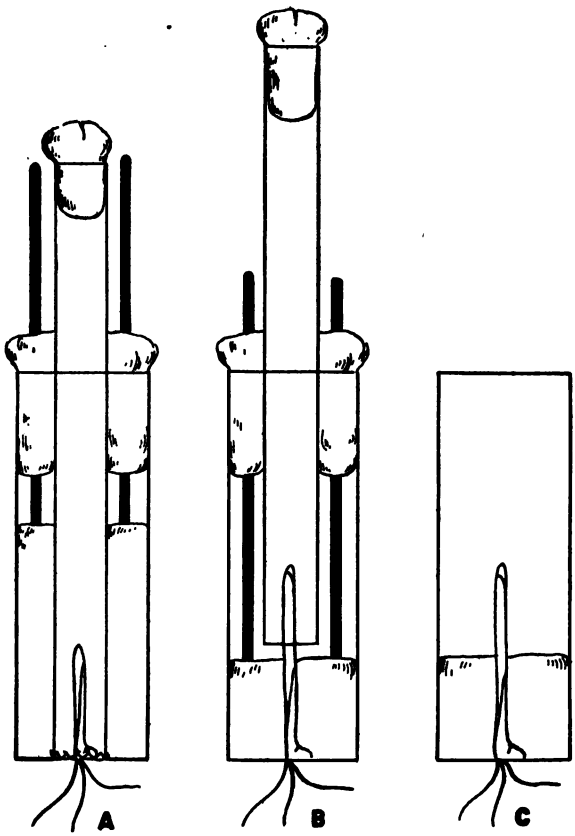


Fig. 13.—Apparatus for growing seedlings free of bacteria.

be repeated two or three times until there is a cotton plug of 3 to 5 cm. around the shoot. Now remove the inner cylinder, excess cotton, and glass rods.

Conversion of Degrees of Temperature on One Scale to Another

$$\text{Degrees C.} \times 1.8 + 32 = \text{Degrees F.}$$

$$\text{Degrees } \frac{\text{F.} - 32}{1.8} = \text{Degrees C.}$$

Comparison of Metric and English Units

Lengths

Millimeters to inches.			Inches to millimeters.		
1	=	0.03937	1	=	25.4001
2	=	0.07874	2	=	50.8001
3	=	0.11811	3	=	76.2002
4	=	0.15748	4	=	101.6002
5	=	0.19685	5	=	127.0003
6	=	0.23622	6	=	152.4003
7	=	0.27559	7	=	177.8004
8	=	0.31496	8	=	203.2004
9	=	0.35433	9	=	228.6005

Capacities

Cubic centimeters to liquid ounces.			Liquid ounces to cubic centimeters.		
1	=	0.03381	1	=	29.574
2	=	0.06763	2	=	59.147
3	=	0.10144	3	=	88.721
4	=	0.13526	4	=	118.295
5	=	0.16907	5	=	147.869
6	=	0.20288	6	=	177.442
7	=	0.23670	7	=	207.016
8	=	0.27051	8	=	236.590
9	=	0.30432	9	=	266.163

Masses

Grams to Avoirdupois ounces.			Avoirdupois ounces to grams.		
1	=	0.03527	1	=	28.3495
2	=	0.07055	2	=	56.6991
3	=	0.10582	3	=	85.0486
4	=	0.14110	4	=	113.3981
5	=	0.17637	5	=	141.7476
6	=	0.21164	6	=	170.0972
7	=	0.24692	7	=	198.4467
8	=	0.28219	8	=	226.7962
9	=	0.31747	9	=	255.1457

Avoirdupois pounds to kilograms.			Kilograms to Avoirdupois pounds.		
1	=	0.45359	1	=	2.20462
2	=	0.90718	2	=	4.40924
3	=	1.36078	3	=	6.61387
4	=	1.81437	4	=	8.81849
5	=	2.26796	5	=	11.02311
6	=	2.72155	6	=	13.22773
7	=	3.17515	7	=	15.43236
8	=	3.62874	8	=	17.63698
9	=	4.08233	9	=	19.84160

Steam Pressures and Their Corresponding Temperatures

Steam pressure. Pounds.	Temperature. Centigrade (°).
0	100.0
1	102.4
2	104.1
3	105.8
4	107.3
5	108.8
6	110.3
7	111.7
8	113.0
9	114.3
10	115.5
11	116.8
12	118.4
13	119.0
14	120.0
15	121.2
20	126.1
25	130.6
30	134.6
40	140.9

International Atomic Weights for 1913[1]

O = 16

Name.	Symbol.	Atomic weight.	Name.	Symbol.	Atomic weight.	Name.	Symbol.	Atomic weight.
Aluminium	Al	27.1	Holmium	Ho	163.5	Rhodium	Rh	102.9
Antimony	Sb	120.2	Hydrogen	H	1.008	Rubidium	Rb	85.45
Argon	A	39.88	Indium	In	114.8	Ruthenium	Ru	101.7
Arsenic	As	74.96	Iodin	I	126.92	Samarium	Sm	150.4
Barium	Ba	137.37	Iridium	Ir	193.1	Scandium	Sc	44.1
Bismuth	Bi	208.0	Iron	Fe	55.84	Selenium	Se	79.2
Boron	B	11.0	Krypton	Kr	82.92	Silicon	Si	28.3
Bromine	Br	79.92	Lanthanum	La	139.0	Silver	Ag	107.88
Cadmium	Cd	112.40	Lead	Pb	207.10	Sodium	Na	23.00
Caesium	Cs	132.81	Lithium	Li	6.94	Strontium	Sr	87.63
Calcium	Ca	40.07	Lutecium	Lu	174.0	Sulphur	S	32.07
Carbon	C	12.0	Magnesium	Mg	24.32	Tantalum	Ta	181.5
Cerium	Ce	140.25	Manganese	Mn	54.93	Tellurium	Te	127.5
Chlorin	Cl	35.46	Mercury	Hg	200.9	Terbium	Tb	159.2
Chromium	Cr	52.0	Molybdenum	Mo	96.0	Thallium	Tl	204.0
Cobalt	Co	58.97	Neodymium	Nd	144.3	Thorium	Th	232.4
Columbium	Cb	93.5	Neon	Ne	20.2	Thulium	Tm	168.5
Copper	Cu	63.57	Nickel	Ni	58.68	Tin	Sn	119.0
Dysprosium	Dy	162.5	Niton	Nt	222.4	Titanium	Ti	48.1
Erbium	Er	167.7	Nitrogen	N	14.01	Tungsten	W	184.0
Europium	Eu	152.0	Osmium	Os	190.9	Uranium	U	238.5
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Gladolinium	Gd	157.3	Palladium	Pd	106.7	Xenon	Xe	130.2
Gallium	Ga	69.9	Phosphorus	P	31.04	Ytterbium (Neoytterbium)	Yb	172.0
Germanium	Ge	72.5	Platinum	Pt	195.2			
Glucinum	Gl	9.1	Potassium	K	39.10	Yttrium	Yt	89.0
Gold	Au	197.2	Praseodymium	Pr	140.6	Zinc	Zn	65.37
Helium	He	3.99	Radium	Ra	226.4	Zirconium	Zr	90.6

[1] Compiled by the International Committee on Atomic Weights, consisting of F. W. Clarke, W. Ostwald, T. E. Thorpe, and G. Urbain.

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